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THE STRUCTURE AND SWELLING PROPERTIES OF *NITELLA* CHLOROPLASTS

By F. V. MERCER,* A. J. HODGE,† A. B. HOPE,* and J. D. MCLEAN*

[Manuscript received August 12, 1954]

Summary

The swelling of the chloroplasts of *Nitella cristata* A. Br. in solutions of different osmotic pressures has been examined in relation to their fine structure, isoelectric point, and permeability towards KCl.

Electron-microscope studies of ultra-thin sections have shown that the normal chloroplast consists of about 40-100 lamellae *c.* 70A apart, enclosed by an external membrane *c.* 70A in thickness. Grana do not occur. Space within the chloroplast not occupied by the lamellae is filled with a granular material resembling the cytoplasm. The chloroplast often contains a considerable number of osmophilic globules *c.* 0.1 μ in diameter lying between the lamellae. Their staining reaction with osmic acid indicates that they are probably lipoidal. Numerous small osmophilic bodies *c.* 100A or less in diameter are associated with the lamellae. Starch grains, when present, occur between the lamellae and appear to be enclosed within by a membrane.

During swelling in hypotonic solutions, chloroplasts change in shape from disc to sphere to cylinder. Swelling results from the formation of vacuoles between the external membrane and the rest of the chloroplast (blebs), by increase in interlamellar spacing and by the formation of vacuoles between the lamellae. The interlamellar vacuoles form by the lamellae rounding up and coalescing to enclose the granular interlamellar material.

Quantitative results for the volume:osmotic pressure relations, the volume:pH relations, the surface charge, and the apparent free space for KCl show that swelling is an osmotic process. The osmotic gradients arise from: (a) a Donnan system associated with the interlamellar material; and (b) from diffusible solutes present in the interlamellar solution. The high values for the apparent free space and the volume:pH relations indicate that the chloroplast membrane is differentially permeable, not semi-permeable.

The degree of swelling of chloroplasts in solutions is controlled by the osmotic pressure and pH of the solution, the differential permeability of the chloroplast membrane and lamellae, and the cohesive forces maintaining the structure of the membrane and lamellae.

By analogy with the chloroplast, it is suggested that the mechanism controlling the water relations of mitochondria, which also possess lamellar structures bounded by a thin membrane, may be similar to that of the chloroplasts.

I. INTRODUCTION

Some of the physical properties of the chloroplast, in particular the swelling properties, have not been satisfactorily explained. Several attempts have been

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made to interpret this property of the plastid in terms of structure. Priestley and Irving (1907) considered that either the peripheral layer of the chloroplast is semi-permeable or that the stroma imbibes water. They tended to favour the latter view because of the difficulty of explaining transfer of metabolites if the former theory were true. Zirkle (1926) denied the existence of a chloroplast membrane and from studies on *Elodea canadensis* Michx. concluded that the expansion of the plastid was due to the uptake of water by a central vacuole probably containing sugar and protein in solution. Hubert (1935) also observed such vacuoles but could not demonstrate their expansion in dilute glycerol.

Knudson (1936) observed the development of bubble-like protrusions (blebs) from the surface of chloroplasts in distilled water and claimed to have caused such blebs to contract in hypertonic sugar solutions. This membrane concept has been supported by Weiler (1936), Granick (1938, 1949), and Strugger (1951). However, Weier (1938), in reviewing the evidence for an outer membrane, points out that while some sort of surface membrane probably exists in the normal chloroplast, it is possible that a membrane with semi-permeable properties forms only when the isolated chloroplast comes in contact with an aqueous phase.

The use of the electron microscope so far has failed to solve the problem although confirming the earlier theory (reviewed by Frey-Wyssling 1948) of a lamellar structure in the chloroplast (see Steinmann 1952a; Cohen and Bowler 1953; Wolken and Schwartz 1953). Electron micrographs of unsectioned chloroplasts have been interpreted as showing an outer membrane (Algera *et al.* 1947; Granick and Porter 1947; Frey-Wyssling and Mühlethaler 1949; Thomas, Bustraen, and Paris 1952). Steinmann (1952b), however, considers that the existence of a well-defined membrane has not yet been shown by direct methods and questions the validity of the assumption that blebs represent portion of the membrane which originally surrounded the chloroplast. Examinations of sectioned plastids in the electron microscope have generally failed to affirm the existence of an outer membrane (Steinmann 1952a, 1952b; Cohen and Bowler 1953; Leyon 1953), although Wolken and Palade (1952, 1953), have shown indications of a surface membrane in sections of *Euglena* sp. chloroplasts.

In view of the inconclusive results obtained by direct observations, the present authors felt that a combination of a quantitative study of swelling properties with light and electron microscopy might yield valuable information. The chloroplasts used for experiments described here were obtained from *Nitella cristata*. This alga proved to be very suitable owing to the ease with which chloroplasts could be isolated from it in an undamaged condition. In the present paper an attempt has been made to relate the swelling properties of the chloroplast to its general structure, surface charge and permeability.

The present studies have resulted in the demonstration of lamellar structure, the absence of grana, and the presence of an external membrane in *Nitella* chloroplasts. They have shown that swelling results either from the formation of vacuoles between the lamellae or from the growth of vacuoles between the

external membrane and the rest of the plastid, or from a combination of these. In both cases the movement of water is controlled by the external membrane and possibly also by the lamellae which may have differentially permeable properties. The external membrane at least is shown to be differentially permeable. Such a membrane would permit the movement of metabolites between the chloroplasts and cytoplasm. The osmotic gradients responsible for swelling arise from the osmotic pressure of solutes in the interlamellar solution and from immobile (Donnan) ions associated with the interlamellar colloids.

II. MATERIAL AND EXPERIMENTAL METHODS

Samples of two varieties of *Nitella cristata* A. Br. were collected from ponds and cultured in their native mud plus tap water in glass troughs. Chloroplasts were isolated usually by excision of one end of an internodal cell (length 2.5 cm) followed by gentle scraping of the cell wall to expel the cell contents. The chloroplasts, which are found packed around the periphery of the cell next to the wall in regular rows, are extruded in apparently undamaged condition.

Ultra-thin sections of *Nitella* cells were prepared for electron microscope examination as follows. Intact cells or swollen isolated chloroplasts were fixed in 2 per cent. OsO_4 , veronal-acetate buffered to pH 7.5 (Palade 1952a), and made up to *c.* 7 atm osmotic pressure with NaCl, for periods up to 48 hr. This was followed by washing, dehydration in alcohol, and embedding in *n*-butyl methacrylate, the general procedures being similar to those of Newman, Borysko, and Swerdlow (1949). Up to 15 per cent. methyl methacrylate was used because of the toughness of the cell walls. Sections between 200 and 300 Å thick were obtained using the technique and microtome of the type described by Hodge, Huxley, and Spiro (1954), and were examined without removal of plastic in a RCA model EMU electron microscope fitted with an externally centerable objective aperture (Farrant and Hodge 1950). The magnification of the instrument was calibrated by the interferometric method of Farrant and Hodge (1948).

In the experiments on swelling in hypotonic media, the chloroplasts from several cells were stored together in the extracted cell sap. Then samples were taken and placed in a relatively large volume of KCl or glucose of known osmotic pressure *p*. The mean volume of at least 50 chloroplasts was calculated from measurements under a high-power microscope ($\times 600$) using a calibrated eyepiece micrometer.

When larger quantities of chloroplasts were required in the investigation of the penetration of KCl, up to 5 g of clean sorted internodal cells were placed in a "Blendor" with 100 ml of glucose of the required osmotic pressure and blended for 5-10 sec. The homogenate was strained through muslin to remove cell wall fragments, and centrifuged at low speed to deposit starch grains. A further spin for 2 min at 150 g produced a residue which was resuspended in fresh glucose, recentrifuged, and the supernatant decanted. This treatment resulted in a substantially pure sample of chloroplasts.

III. RESULTS

(a) Description of the Swelling Process

(i) *The Normal Chloroplast.*—*In vivo*, under the light microscope or the phase contrast microscope, the chloroplasts appear as pale green, disc-shaped bodies, of $5\text{--}10\ \mu$ dia. by $1\text{--}2\ \mu$ thick. Starch grains are readily visible. In surface view most chloroplasts appear green but not optically homogeneous. Frequently, many had a finely spotted appearance, the spots being circular, of about $0.3\ \mu$ dia. and darker in colour than the rest of the chloroplast. The spots were similar in size to the grana of some other chloroplasts (Strugger 1951). Plate 1, Figure 1, shows chloroplasts viewed from above in the living cell, compared with those isolated in 0.5M glucose (Plate 1, Fig. 2). The significance of the spotted appearance will be discussed later in relation to the chloroplast fine structure. In side view (Plate 1, Fig. 3), they appeared approximately elliptical when isolated.

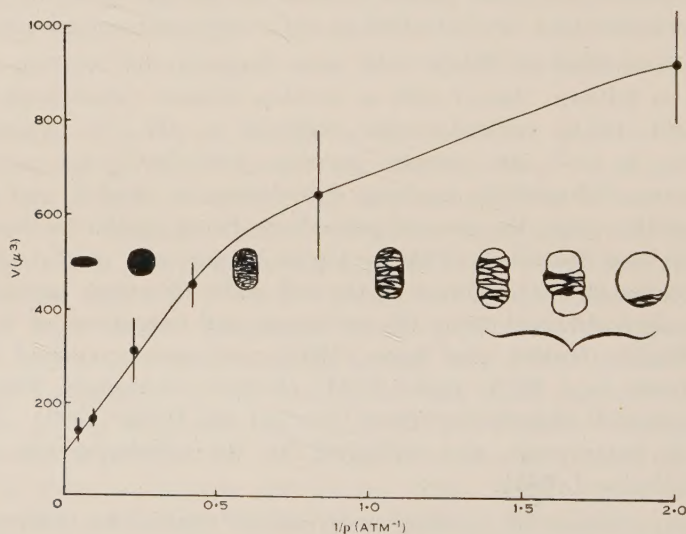


Fig. 1.—Mean volume V in μ^3 of a large number of *Nitella* chloroplasts plotted against the reciprocal of the osmotic pressure of the KCl solution ($1/p$) in reciprocal atmospheres. Each point represents the mean and standard deviation of independent samples taken from cell sap. The diagrams represent the mean appearance of the chloroplasts at the osmotic pressure below each drawing.

(ii) *Swelling in Hypotonic Solutions.*—As it was not possible to follow the swelling process in a single chloroplast, the morphological changes associated with swelling have been reconstructed from the mean changes observed in many chloroplasts. Although there was variation in the degree of morphological change which occurred, particularly in the very dilute solutions, swelling followed a definite pattern.

As illustrated in Figure 1, a chloroplast changes from disc to sphere to cylinder, and the latter continues to expand, ultimately forming large vacuoles or blebs, though a single spherical bleb is often observed. Most of the change in volume results from an expansion along the initial minor axis of the chloroplast. Over the concentration range 0.05-0.1M approximately, the chloroplast increased in volume, becoming spherical. During the disc to sphere transformation the general appearance of the chloroplast remained constant. The sphere-cylinder change between 0.1M and distilled water was associated with gross structural changes. As the cylindrical form developed, the striations became more obvious as alternating dark green and light bands. With further swelling the dark green striations appeared as a coarse reticulum with colourless vacuole-like regions between the meshes. The strands of the meshwork ranged from *c.* 2 μ to the limits of resolution of the microscope in width. At this degree of swelling a distinct membrane could be seen enclosing the reticulum.

The increase in volume resulted mainly from the increase in the vacuole regions rather than from an increase in the volume of the reticulum. Frequently blebs of up to 20 μ dia. developed from the surface of the chloroplast. Where one or two large blebs were formed from a plastid they were most commonly developed next to the hemispherical ends of the plastids. Examples of the vacuoles and blebs are given in Plate 2, Figures 4, 5, and 6.

Attempts to reverse the swelling process were not successful. This is contrary to the results of Knudson (1936) who succeeded in causing blebs to contract in hypertonic solutions. No structures identifiable as grana were observed during swelling.

Further information concerning structural changes associated with swelling was obtained from electron-microscope studies of normal chloroplasts *in situ* and of isolated chloroplasts showing different degrees of swelling.

(b) The Fine Structure of *Nitella* Chloroplasts

The general morphological features of *Nitella* are shown in Plate 3, Figure 7, which is of a section cut transversely to the major axis of a cell kept in the dark for 10 days to minimize the formation of starch grains. Starch grains, which do not stain well with OsO_4 , usually swell during the embedding procedure and cause distortion. Starch-free cells were therefore usually selected for examination.

The first feature of interest in Plate 3, Figure 7, is the cell wall which exhibits a number of striations, most clearly defined in the region adjacent to the cytoplasm. These resemble the growth rings seen in cotton fibres (see references in Hock 1942). The disc-shaped chloroplasts are closely applied to the cell wall and frequently overlap to some extent. The lamellar structure of the chloroplasts is evident, as is the chloroplast limiting membrane. In places the membrane is separated from the lamellae, the intervening space being filled with some osmiophilic material (see also Plate 4, Figs. 9 and 10). In the bifurcations between the groups of lamellae are located starch grains or starch vacuoles and a number of spherical densely staining bodies of fairly uniform size (0.1-0.2 μ). Similar bodies are shown in the micrograph of a chloroplast

of *Aspidistra* published by Finean, Sjöstrand, and Steinmann (1953). The spherical shape and osmiophilia suggest that they may be oil globules. The tonoplast, separating the central vacuole and cytoplasm, appears as a thin (*c.* 70 Å) membrane of varying width. This appearance is characteristic of thin membranes which are oriented at angles less than 90° to the plane of the section. The tonoplast is better seen in Plate 4, Figure 10.

The lamellar structure of the chloroplast is shown more clearly in Plates 4-6. The lamellae are seen as thin dark lines about 40 Å thick, separated by less dense zones about 30 Å thick. The lamellae extend continuously across the entire width of the chloroplast. Stacking is more regular towards the edges of the chloroplast, where up to 40 lamellae may be seen in almost perfect array. In the more central regions of the chloroplast, groups of lamellae are separated by spaces of variable width, in the larger of which are located the starch grains or vacuoles and the dense spherical bodies described above. The existence of these spaces between groups of lamellae and their shape may offer an explanation of the inhomogeneous spotted appearance of the chloroplasts when viewed under the light microscope. Since several of the dense-staining, spherical bodies appear within each chloroplast in thin sections, it is clear that the total number of spaces must be large. Further, if the refractive index of the material filling these spaces differs appreciably from that of the lipoprotein lamellae, the spaces will show up in surface view as inhomogeneities under the light microscope.

The periodic spacing observed in the well-ordered regions of lamellae is somewhat variable, ranging from less than 70 Å to about 120 Å. Some of this variation is probably intrinsic, i.e. due to variation in the perfection of packing in the intact chloroplast and to the thickness of the interlamellar zones, but some is undoubtedly due to variation in the angle of sectioning. It therefore seems likely that the true spacing, consistent with that to be expected for a single lipoprotein layer structure, in *Nitella* is in the vicinity of 70 Å, a value very much less than has been reported for chloroplasts of other plants (Cohen and Bowler 1953; Finean, Sjöstrand, and Steinmann 1953; Wolken and Schwertz 1953). The regular structure of *Nitella* chloroplasts suggests that this alga would be suited to X-ray diffraction work, especially at small angles.

The presence of grana in the chloroplasts of higher plants has been confirmed by electron microscopy (Steinmann 1952*b*; Cohen and Bowler 1953; Finean, Sjöstrand, and Steinmann 1953). The grana are sharply defined regions of well-ordered lamellae with a periodic spacing of about 70 Å. Such differentiation is absent from the chloroplasts of *Nitella*. Since the lamellar spacing is of the same order as that observed in the grana of higher plants, the *Nitella* chloroplast might be regarded as a single giant granum.

Plates 3-6 show clearly the limiting membrane surrounding the chloroplast. This membrane appears to be about 70 Å thick, although such measurements on a single membrane are necessarily inaccurate. In Plate 4, Figure 8, the membranes of two adjacent chloroplasts can be seen closely applied to each other.

The larger spaces within the chloroplast not occupied by the lamellae are filled with a relatively homogeneous granular material. In appearance it re-

sembles the cytoplasm and might appropriately be termed chloroplasm. In addition, numerous small very dense granules of irregular shape (mostly of less than 100 Å dia.) are present, associated mainly with the lamellae, and probably represent small localized deposits of osmium (Plates 4-8). The association of these granules with the lamellae is well seen in the swollen plastids (Plate 8, Fig. 16). It is possible that they result from the reduction of OsO_4 by some reducing system within the lamellae. Numerous reducing systems, e.g. ascorbic acid, reduced coenzymes, and carotenoids, are associated with the chloroplast (Weier and Stocking 1952). It is of interest that similar dense granules were observed by Sjöstrand (1953) within the outer segments of retinal rods, which have a lipoprotein layer structure somewhat similar to that of chloroplasts.

Plate 6, Figure 13, shows a number of morphological features of interest, in addition to those already described. On the right are three cytoplasmic inclusion bodies which are probably mitochondria. They possess thin limiting membranes and exhibit internal structures resembling the cristae of animal mitochondria described by Palade (1952*b*). In the upper right are two cytoplasmic "vacuoles." The membranes of these "vacuoles" are closely applied to the tonoplast membranes, resulting in a double membrane effect. The cytoplasm has a reticular appearance and many lamellar structures are present, often in close apposition. These are identified as elements of the endoplasmic reticulum (Palade and Porter 1952). The lamellar elements (or flattened canalicular elements) are also evident in Plate 4, Figures 9 and 10, and Plate 5, Figure 11.

It has been suggested (Zirkle 1926) that starch forms in specialized starch vacuoles within the chloroplast. The results obtained here are not inconsistent with this hypothesis. Plate 5, Figure 12, shows a region of low density surrounded by what appears to be a thin closed membrane located in the space between two bands of ordered lamellae. Since some starch appeared to be present within the enclosed region (not clearly seen in the illustration because of contrast limitations), this structure is tentatively identified as a starch vacuole.

The cytoplasm of *Nitella* is clearly limited on one side by the tonoplast membrane, but a cell membrane between the cytoplasm and the cell wall has not yet been observed in the sections. This may be due to its thinness and close apposition to the cell wall or there may be no membrane in this position. The chloroplast membrane is also difficult to observe on the cell wall side.

Thin sections of swollen chloroplasts show clearly the nature of the structural changes taking place during the disc-sphere-cylinder transformation. The initial stage (disc-sphere) involves mainly an increase in the average spacing of the lamellae. However, wide variations exist, and the general pattern is often complicated by the formation of large blebs and vacuolar regions between the bands of lamellae. Plate 7, Figure 14, illustrates a chloroplast which is at a stage of swelling similar to that shown optically in Plate 2, Figure 4. The lamellar organization is still quite well preserved, but the chloroplast membrane has formed two large blebs at the ends. The membrane is cut obliquely and therefore appears as a thin ribbon. The interlamellar spacing here is about 500 Å in the plane of the section. As swelling progresses the lamellae coalesce to

form small spherical vacuoles. Plate 7, Figure 15, shows a cylinder stage in which vacuolation has occurred, although stacks of lamellae still remain.

In greatly swollen chloroplasts the structure is completely vacuolated (Plate 8, Fig. 16). As is evident in this section the membrane of the chloroplast is usually still intact. The interior of the chloroplast is now filled entirely with spherical vacuoles, each enclosed by a thin membrane formed by fusion of lamellae. A starch grain enclosed in a membrane can also be seen. The very small dense granules described earlier are located on the lamellae (Plate 8, Fig. 16) and the vacuoles contain a reticular material. This presumably represents the remains of the chloroplast or interlamellar material, and its appearance is consistent with denatured precipitated protein. The transformation of the lipoprotein lamellae to enclose spherical vacuoles indicates the extreme lability of these structures, and suggests the necessity for caution in the interpretation of lipoprotein systems which have been exposed to hypotonic solutions.

Samples of swollen chloroplasts were also examined in the electron microscope using the conventional method of drying a suspension on a collodion-covered specimen screen, washing several times with distilled water, and shadow-casting with uranium. Plate 8, Figure 17, is a typical example of such a preparation. The appearance of this micrograph is entirely consistent with that of Plate 8, Figure 16. The outer limiting membrane is clearly defined, as are a number of flattened vacuoles in varying size. The outer limiting membrane, presumably of double thickness as expected if a spherical bleb is flattened onto a surface, is about 150 Å thick as measured by the length of a shadow. This value is in good agreement with the single membrane thickness (*c.* 70 Å) determined from thin sections. The vacuoles, trapped between the two layers of the chloroplast membrane, throw sharp shadows of a length similar to that of the chloroplast membrane. The thickness of the single vacuole membranes measured in this way is also about 70 Å.

(i) *The Nature of the Swelling Process.*—The results obtained with the light microscope and the electron microscope show that the overall volume changes which occur in a chloroplast in solutions of various tonicity are determined by volume changes in the interlamellar regions and in the vacuoles which form within the chloroplast.

Information on the nature of the processes controlling these volume changes was obtained by studying the water relations of chloroplasts under various experimental conditions.

(c) *The Water Relations of Isolated Chloroplasts as a Function of Osmotic Pressure*

Nitella chloroplasts, extracted and kept in cell sap of approximately 12 atm osmotic pressure, were placed in KCl solutions of various osmotic pressures and the mean volume measured. This was done with an eyepiece micrometer of which one small division was approximately 2 μ in the object. If the chloroplasts were platelets, they were treated as oblate spheroids and the major and

minor axes of a number were measured. Measurement of diameter sufficed when the shape was spherical and the much elongated or sausage-shaped chloroplasts in very dilute media were treated as cylinders.

It was found that the mean volume was inversely proportional to the osmotic pressure p of the medium over a large range of p . Figure 1 shows volume plotted against $1/p$ for KCl solutions 0.5-0.01M ($p = 22 - 0.5$ atm). Each point is the mean and standard deviation for five experiments in each of which 50 chloroplasts were measured. Figure 1 shows that between KCl concentrations of 0.5 and 0.05M, the experimental relation between the volume V and p satisfies the Boyle-van't Hoff law:

$$p(V - b) = \text{const.}, \quad \dots \quad (1)$$

where b is a "non-osmotic volume" corresponding to the volume at infinite p . This is approximately $90 \mu^3$ in the variety used in the experiments of Figure 1. Swelling in glucose followed the same general pattern.

Reasons for the non-linearity of the $V : 1/p$ graph in Figure 1 for $c_0 < 0.05M$, where a halving of the osmotic pressure does not produce a doubling in volume, are discussed in Section IV.

Thus the chloroplast contains either a system comparable to the protoplast (Levitt, Scarth, and Gibbs 1936), i.e. an aqueous (vacuolar) phase surrounded by a semi-permeable membrane, or a gel-like structure, or both. In the former, swelling is due to osmotic pressure differences between the media and a solution of sugars and possibly salts, maintained by a semi-permeability of the separating membrane. In the second system osmotic pressure differences are due to the unequal distribution of ions as a result of the Donnan effect of fixed charges on protein molecules in the gel system. Experiments are to be described which bear on this question.

(d) Electrophoresis of Isolated Chloroplasts

The electrophoretic mobility of *Nitella* chloroplasts, isolated as described in Section II, in glucose + buffer solution was measured as a function of pH in a cell of special design.*

The results are shown in Figure 2 in which electrophoretic mobility in $\mu \text{ sec}^{-1}/V \text{ cm}^{-1}$ in buffers of ionic strength 0.01 is plotted against pH of the buffer solution. It is seen that migration is towards the cathode (positive charge on the chloroplasts) at pH values less than 4.2 and towards the anode at pH values more alkaline than this. The isoelectric point of the surface of these bodies is thus at pH 4.2 in sodium acetate-acetic acid buffer. This is similar to the isoelectric point of the "chlorophyll-protein complex" measured by Fishman and Moyer (1942). The mobility values have been corrected for the viscosity of the glucose solutions. Table 1 gives charge density σ for a number of pH values, calculated according to Abramson and Moyer (1936). The charge density at near neutral reaction is similar to that calculated by these authors

* A fuller account of the electrophoresis of chloroplasts and other cell components will be published later.

for various blood cells, but the isoelectric point is much higher. Red cells are isoelectric at pH *c.* 1.7 (Furchgott and Ponder 1941).

The electrophoretic behaviour of the isolated chloroplasts is thus consistent with the presence of a protein or lipoprotein component at the surface. It is unlikely to be a phospholipid as these commonly have an isoelectric point at pH *c.* 3 (Frey-Wyssling 1948). The possibility that the results are due to the adsorption of a cytoplasmic protein retained after isolation cannot be dismissed, although repeated washing, centrifugation, and resuspension in fresh buffered glucose did not alter the results.

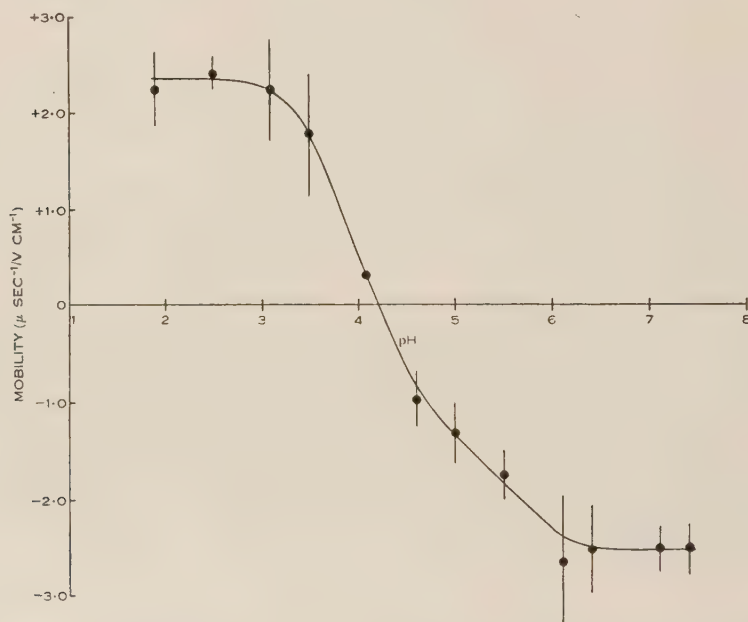


Fig. 2.—Mean mobility v in $\mu \text{ sec}^{-1}/V \text{ cm}^{-1}$ plotted against pH of buffer solutions for electrophoresis of *Nitella* chloroplasts. Each point is the mean and standard deviation of a number of measurements.

The mobilities and isoelectric point were also unaltered after treatment with 1 per cent. solution of crystalline trypsin, buffered to pH 8 for 2 hr at 37°C. The chloroplasts appeared normal under the light microscope after such treatment. Thus the component of the chloroplast responsible for the electrophoretic behaviour described above appears to be resistant to the proteolytic enzyme trypsin, or else this component is continuous from the surface inwards, the trypsin merely removing some of the external molecules. However, prolonged trypsin treatment left the chloroplast unaltered except for a disorganization attributable to the alkalinity of the medium. The experiment will be repeated with pepsin and lipase.

The possible existence of a three-dimensional system of fixed charges (Donnan system) will now be considered in regard to the water relations of the chloroplast as a function of pH.

(e) The Water Relations of Isolated Chloroplasts as a Function of pH

When samples of chloroplasts were placed in 0.1M KCl buffered with McIlvaine's (citric acid + disodium hydrogen phosphate) buffer, the mean volume was found to be a function of pH as shown in Figure 3. At a pH in the vicinity of 4 the volume is less than at more acid or alkaline reactions. This effect is similar to that obtained with gelatin (Loeb 1921-22) and supports the alternative mentioned previously that swelling may result from osmotic pressure differences caused by a Donnan distribution of ions. If this is so, the swelling would be expected to be a minimum at the isoelectric point where the net concentration of immobile ions is zero. With the chloroplast a minimum of swelling was found at pH 4, which is close to the isoelectric point of the external surface material and that of the fragmented chloroplast. It seems reasonable to suppose from this evidence also that a material isoelectric at pH c. 4 (protein or lipoprotein) extends throughout the chloroplast. It should be noted that the mean volumes in Figure 3 should not be compared with those of Figure 1 since a different variety of *Nitella* was used in these experiments. For example, the "non-osmotic volume" *b* of this variety is $50 \mu^3$ and therefore the volume at pH 4 is much greater than the "non-osmotic volume."

If the swelling process in the chloroplast is similar to that found in the mature plant cell, then the size of the osmotic gradient between the interior and exterior of the chloroplast will be a major factor in controlling the degree of swelling in solutions. The size of the gradient will be influenced by the permeability of the chloroplast and the chloroplast membrane. Data bearing on this point were obtained from measurements of the apparent free space of the chloroplast.

TABLE 1
SURFACE CHARGE DENSITY σ AS A FUNCTION OF pH IN *NITELLA* CHLOROPLASTS

pH	3.1	3.5	4.1	4.6	5.5	6.25
σ (e.s.u./cm ²)	2411	1841	317	-957	-1795	-2455

(f) The Apparent Free Space of Isolated Chloroplasts

The apparent free space of the chloroplasts or the percentage of the plastids which appears to reach equality of concentration with the medium by diffusion was calculated as described in Hope and Stevens (1952), from measurements of conductivity of the medium. Approximately 80-90 per cent. by weight of the chloroplast pellet is apparent free space and this value does not seem dependent on the KCl concentration or osmotic pressure of the medium.

Chloroplasts, from a second variety of *Nitella cristata* because of its greater abundance, were isolated as described in Section II in glucose. The final result was a pellet of chloroplasts 0.05-0.1 g in weight which was mopped of excess water although some remained packed with the chloroplasts. This excess water results in an over-estimation of the apparent free space due to dilution of the medium, and may account for 30 per cent. of the change in conductivity

when the chloroplasts are spheres but less when they are oblate spheroids ($p > 4$ atm). Exchange adsorption was not expected to change the conductivity appreciably. 5 ml of KCl or KCl + glucose were added and readings of conductivity made of samples of the supernatant after centrifugation for 2 min at 150 g. After correction for water held in the pellet, the apparent free space is approximately that given in Table 2.

As the equilibration between chloroplast and medium is completed before the first possible reading (at 3 min) it is not possible to estimate the diffusivity of KCl in the chloroplast material except that it is much greater than 2×10^{-9} cm² sec⁻¹ (cf. 1.7×10^{-5} cm² sec⁻¹ for 0.1M KCl in H₂O at 20°C). This

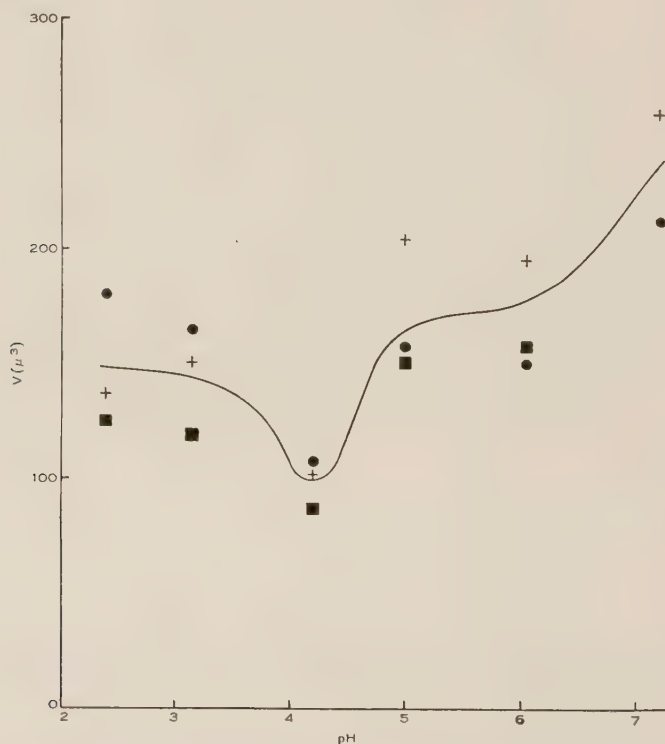


Fig. 3.—Mean volume V in μ^3 of isolated *Nitella* chloroplasts, plotted against pH of buffer solutions.

value is calculated from Fick's equation (see Davson 1943) by assuming diffusion into a sphere of radius 5μ and $c_i/c_0 = 0.99$ at $t = 180$ sec.

Summarizing, these results show that most of the chloroplast volume can be penetrated by KCl in a short time and that the chloroplast membrane is permeable to KCl.

IV. DISCUSSION

The results show that over a wide range of hypotonic solutions the mean volume of the chloroplast of *Nitella* is proportional to the reciprocal of the

osmotic pressure of the solutions. The relationship $V \propto 1/p$ is usually attributed to the swelling of an aqueous phase enclosed by a semi-permeable membrane (Höber 1945). On the other hand, the swelling of a gel system because of the Donnan distribution of ions may also satisfy this relationship (Ponder 1946). Compliance with the Boyle-van't Hoff law does not distinguish between the two types of systems. The only conclusion justified is that osmotic forces, caused either by the presence of a semi-permeable membrane or by a gel system or both, control the swelling of the chloroplasts.

The importance of the Donnan system is suggested by the relationship between swelling and pH, and also by the presence of the coagulated interlamellar material in the vacuoles. Further, as swelling shows a minimum at approx. pH 4, which is also close to the isoelectric point of the chloroplast surface and "chlorophyll-protein complex," proteins or lipoproteins could be thought to form the non-diffusible ions of the Donnan system. As chloroplasts contain c. 40-50 per cent. protein on a dry weight basis (Menke 1938; Frey-Wyssling 1948), there is sufficient protein present to yield a large number of fixed charged groups. Additional support for a Donnan system is shown by the apparent free

TABLE 2
* APPARENT FREE SPACE OF *NITELLA* CHLOROPLASTS IN KCl AND KCl + GLUCOSE

KCl (mol/l)	Glucose (mol/l)	Osmotic Pressure (atm)	Apparent Free Space (%)
0.05	0.32	10	8†
0.05	—	2.5	70, 66, 71
0.10	—	4.6	87, 80

space data for KCl. If the permeability for the ions of the buffer system used in the isoelectric point experiments is similar to that for KCl, the pH effect most probably operates on substances within the structure of the chloroplast.

At least two structural arrangements within the chloroplast could result in a Donnan system. Either the non-diffusible ions are prevented from diffusion by a membrane, or they are fixed in a three-dimensional structure, or both. Although the electron micrographs show that swelling results from an increase in the volume of the interlamellar regions, they do not demonstrate the relative distribution of the polyvalent ions between the lamellae and the interlamellar regions. The size of most vacuoles would exclude Donnan forces arising from fixed ions in the lamellae, except during the initial stages of the disc-sphere transformation. Donnan forces resulting from ions attached at a surface would not exert an influence on water molecules beyond c. 10 Å from the surface. The vacuoles contain a reticular material which is probably derived from the interlamellar substance. This material, which appears to disperse throughout the vacuole during swelling, may provide free polyvalent ions. As the lamellae and the external chloroplast membrane appear to be impermeable to

this substance, a Donnan system would exist; in this case, the water associated with the polyvalent ions would be dispersed throughout the vacuoles.

The quantitative results on swelling do not permit a distinction to be drawn between these possibilities. An analysis of the fine structure of the chloroplasts favours the view that the interlamellar material rather than the lamellae is the site of the Donnan ions. If the structure of the lamellae is similar to that of the lipoprotein layers in the myelin sheath (Finean 1953) the protein component of the lamellae may not be able to contribute fixed ions because it is screened from the aqueous phase by the lipid component. If this is so, the protein of the interlamellar material would be the dominating factor. Wolken and Schwartz (1953) have postulated that the lipid and protein are arranged in alternate layers, with the hydrophilic porphyrin heads of the chlorophyll molecules in the interface between the lipid and the aqueous protein layer. However, by analogy with the probable structure of myelin (see Finean 1953 for references), it seems more likely that the lamellae comprise *c.* 30 Å layers of protein, with the lipid molecules oriented with axes normal to the protein

TABLE 3

THEORETICAL OSMOTIC PRESSURE DIFFERENCES δp BETWEEN IONIZED GEL (NON-MOBILE ION CONCENTRATIONS A) AND MEDIUM, AND ACROSS A SEMI-PERMEABLE MEMBRANE, FOR EXTERNAL CONCENTRATIONS c_0

c_0 (mol/l):	0.5	0.25	0.1	0.05	0.025	0.01
δp (atm) for:						
$A = 0.02M$	0.0048	0.0072	0.024	0.048	0.092	0.199
$A = 0.10M$	0.12	0.24	0.86	1.00	1.48	1.97
Semi-permeable membrane	0	11.0	17.1	19.1	20.2	22.0

layers on one or both sides. Aqueous phases would exist between the hydrophilic surfaces of such compound layers. The phytol chains of chlorophyll could penetrate the lipid layers, the hydrophilic porphyrin heads remaining in the aqueous phases. The important distinction between these two views is that in the scheme of Wolken and Schwartz (1953), all of the protein of the chloroplast is in the aqueous phase, thus contributing Donnan ions, while the latter view suggests that the protein of the lamellae may not contribute to the Donnan effect nor to the apparent free space for KCl. Rather the protein of the aqueous phase, i.e. the interlamellar material, would fulfil this function.

Although the evidence demonstrates the importance of Donnan effects, it can be shown that the theoretical osmotic pressure differences due to the Donnan system would account for only a small fraction of the total volume changes observed.

As a first approximation consider the chloroplast, or that part concerned with swelling, as a homogeneous gel containing A mol/l of monovalent cations adsorbed to fixed negative valencies, of number n per protein molecule; i.e. the

protein concentration is A/n . Consider the simpler case where ionic equilibrium occurs without swelling, as this enables us to calculate osmotic pressure differences without the complication of having a variable A .

If the adsorbed cations are all K^+ ions and the medium is KCl, it can be shown that

$$c_i = \frac{1}{2} [(A^2 + 4c_0^2)^{\frac{1}{2}} - A], \quad \dots \quad (2)$$

where c_i is the concentration of "extra" K^+ and Cl^- ions at equilibrium as a function of A and external concentration c_0 . Assume further that n is very large (molecular weight of the protein high) so that the contribution of the protein itself to the osmotic pressure is negligible. Then the osmotic pressure difference between chloroplast and medium is given by

$$\delta p = RT [(A^2 + 4c_0^2)^{\frac{1}{2}} - 2c_0]. \quad \dots \quad (3)$$

Now a value of A for calculation purposes can be arrived at as follows. The net negative charge on the external surface at pH 6-7 may mean the presence of a three-dimensional array of protein chains or lattices with similar charge distribution. In such a case at pH 6.25, σ (charge density of surface) = 2455 e.s.u./cm² (see Table 1), corresponding to 5.11×10^{12} unit charges/cm², and therefore to 11.55×10^{18} /cm³. Thus $A \simeq 0.02$ "mol"/l of such negative charges. Non-diffusible anion concentrations of this order of magnitude were calculated for the surface and interior of root cell cytoplasm (Hope 1953).

Using (3), values for δp for various c_0 values are calculated in Table 3 for $A = 0.02$, 0.1M, and for a semi-permeable membrane + "vacuole" phase arbitrarily made isotonic with $c_0 = 0.5M$ KCl. Thus it is seen that the osmotic pressure differences between the chloroplast phase and the medium, when considering a Donnan system, are less by more than an order of magnitude than those present across a semi-permeable membrane. Only if A is very much greater than indicated by the surface charge density could such a mechanism account for volume: osmotic pressure relations such as in Figure 1. If $A = 0.1M$, however, an appreciable swelling tendency would be present owing to Donnan effects.

Thus it seems likely that Donnan forces could not account for all the swelling of chloroplasts in hypotonic solutions. As swelling obeys the Boyle-van't Hoff law, it follows that the main volume changes must arise from an osmotic system consisting of solutes enclosed by a membrane. The fine structure of the chloroplast is consistent with this conclusion. The chloroplast is enclosed by a membrane and the fused lamellae form membrane-like structures. As both the lamellae and the external membrane are permeable to KCl, they are probably also permeable to other small solutes. Hence the interlamellar regions would probably be in equilibrium with the solutes of the cytoplasmic sap. In addition metabolites concerned with photosynthesis would also occur in the interlamella. When the chloroplast was transferred to an external solution water would diffuse into or from it according to the direction of the osmotic gradient between the interior and exterior. The degree of swelling which occurred would depend on the relative rates of movement of water and the solutes.

Several experimental results support this view. Conspicuous vacuoles do not form when chloroplasts are transferred to water by gradually diluting the external solution. Also the departures from the ideal $p : V$ relationships observed are to be expected since swelling would vary according to the rate of loss of solutes from the chloroplast.

The non-reversibility of swelling may indicate that the membranes become completely permeable when stretched; or it may mean that the lipoprotein system becomes disorganized when much swollen.

It is concluded that both a Donnan system and osmotic pressure due to diffusible solutes are responsible for the water relations of the chloroplast. A similar explanation could account for the water relations of mitochondria, particularly since as shown by the electron microscope data they also consist of a laminated system enclosed by a membrane.

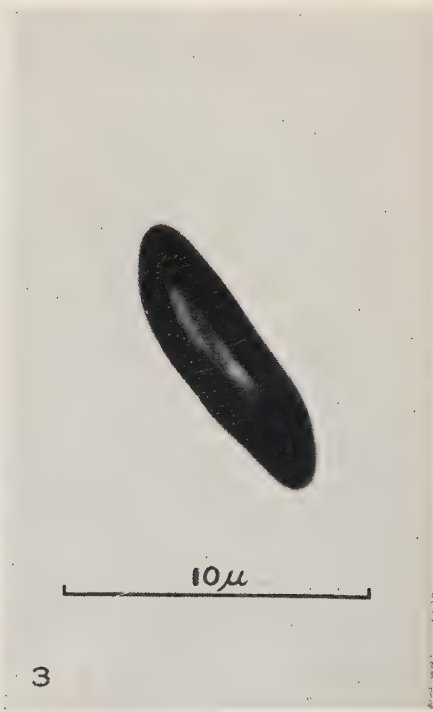
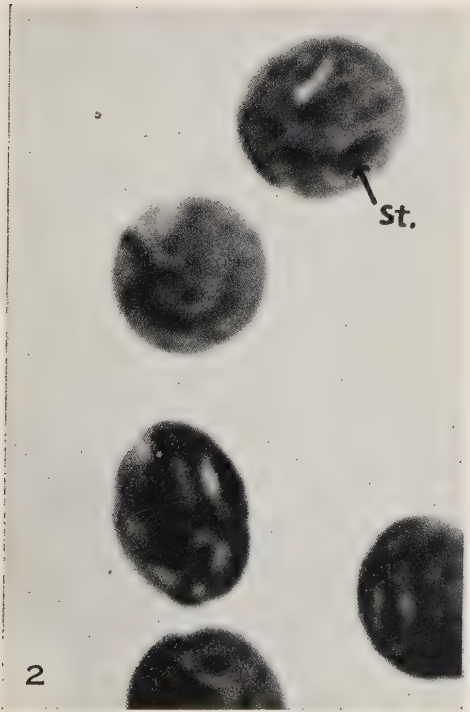
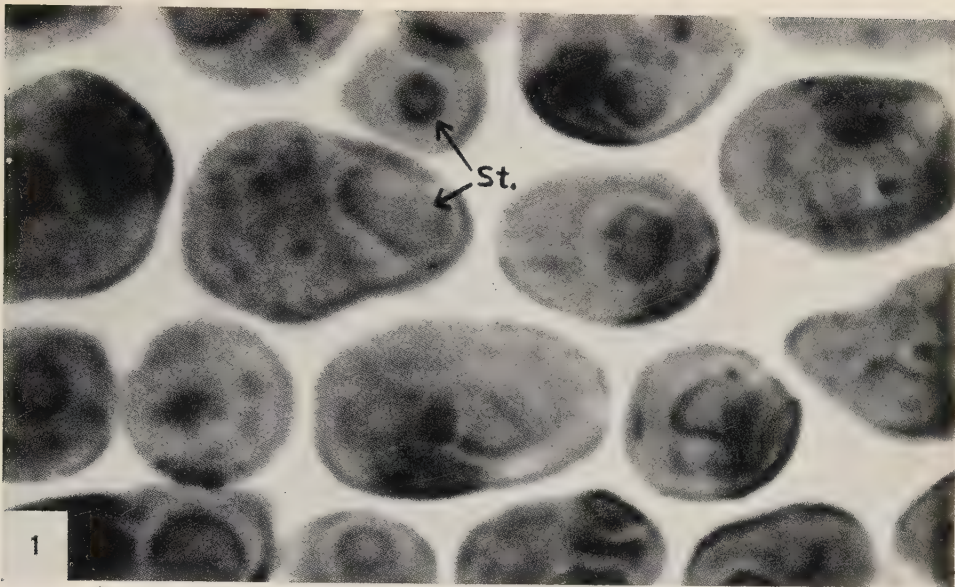
V. ACKNOWLEDGMENTS

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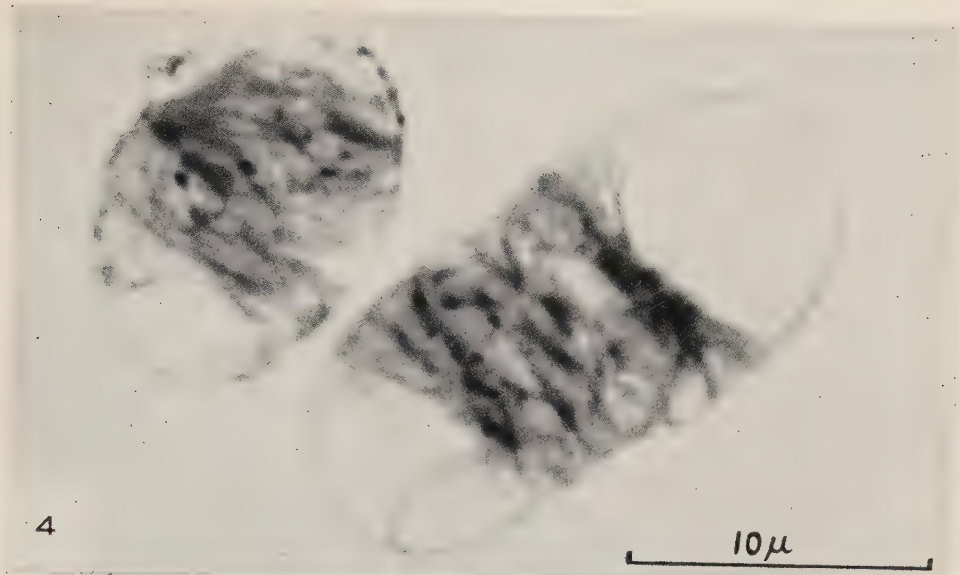
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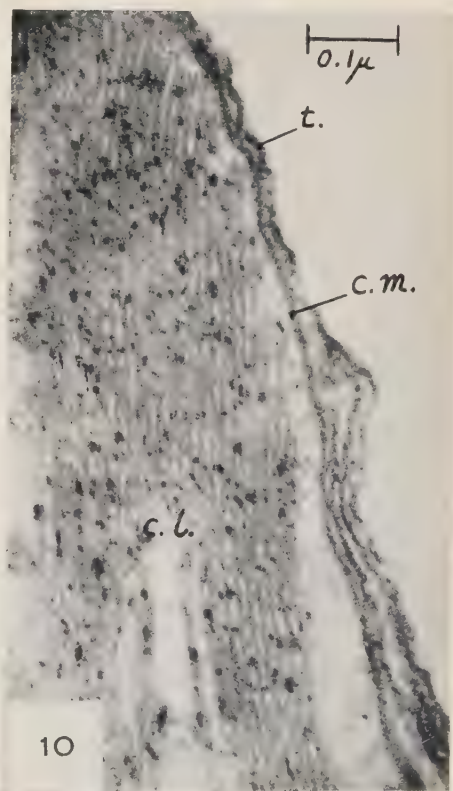
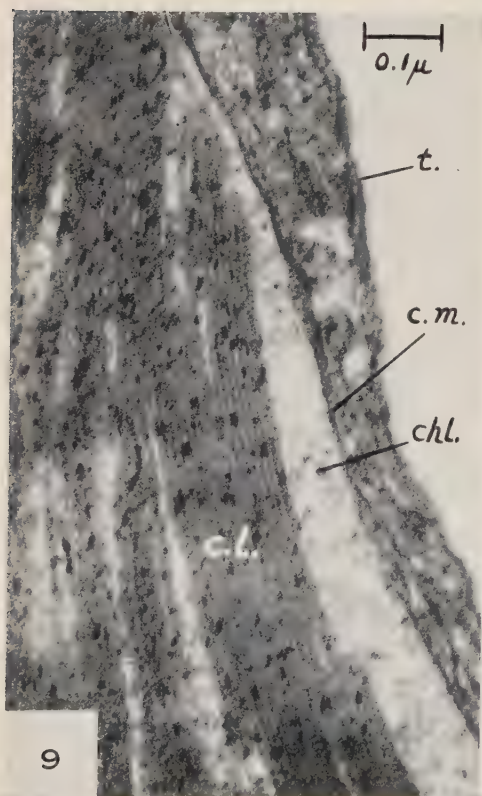
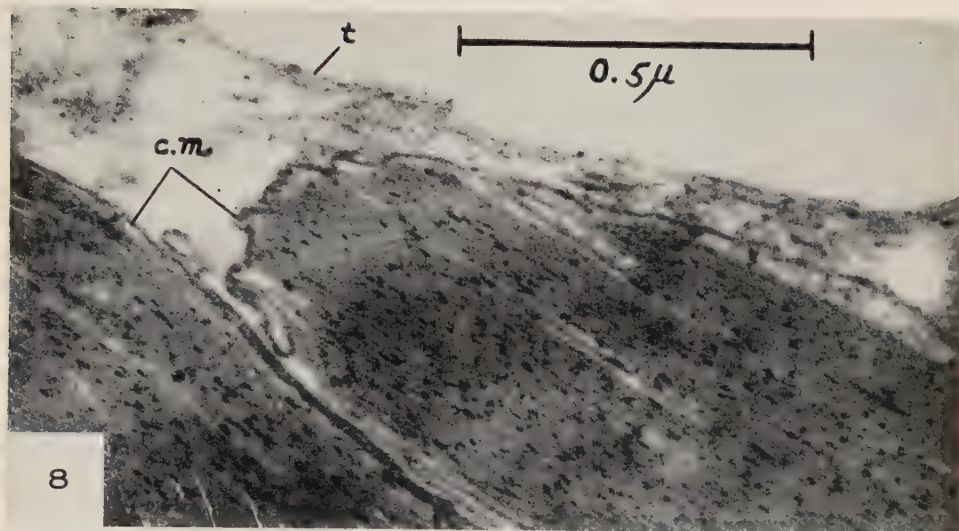
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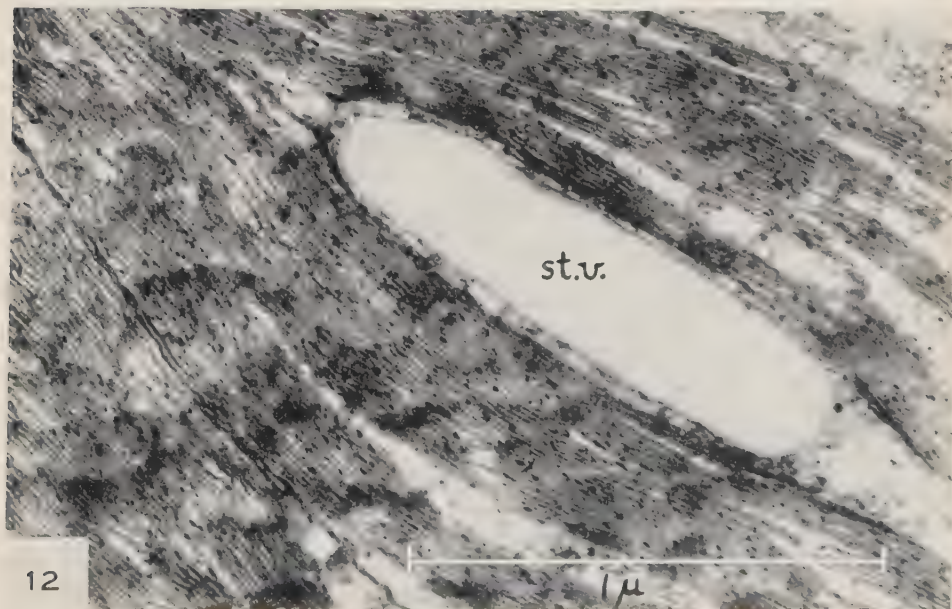
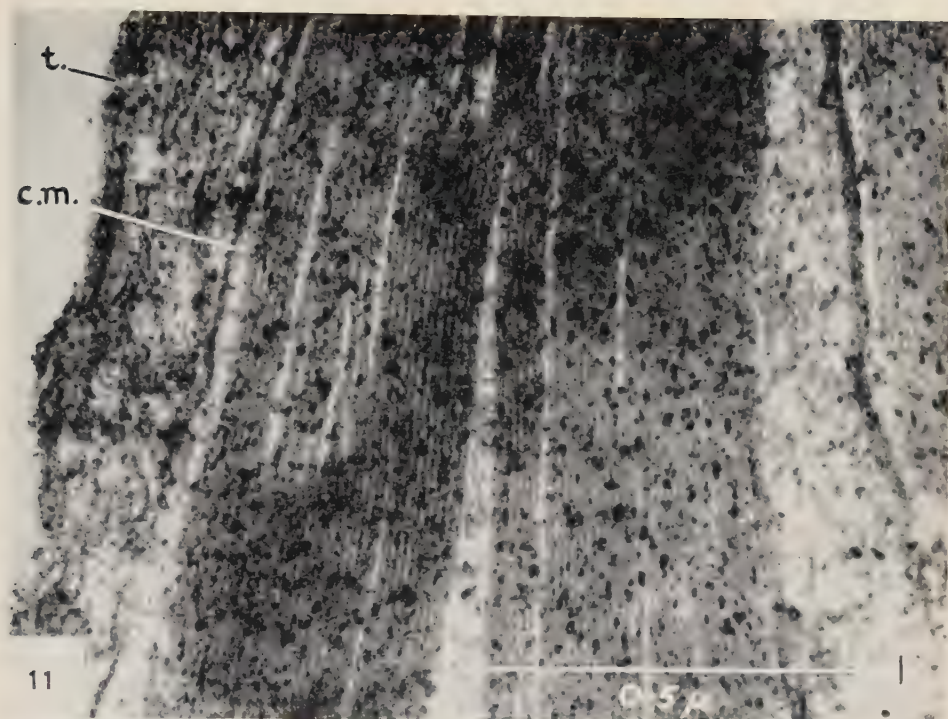
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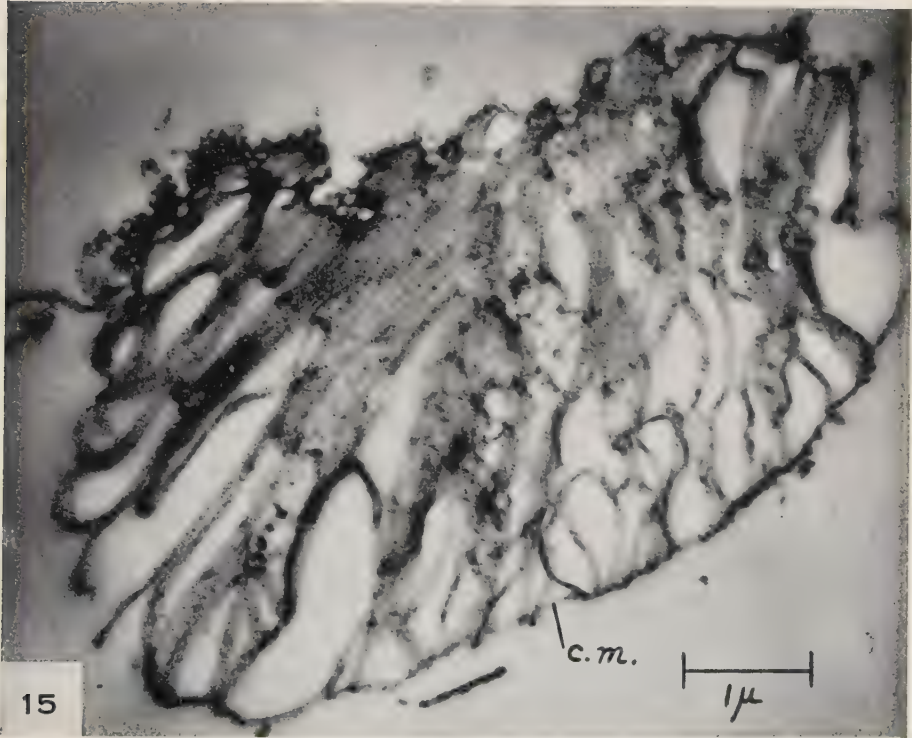
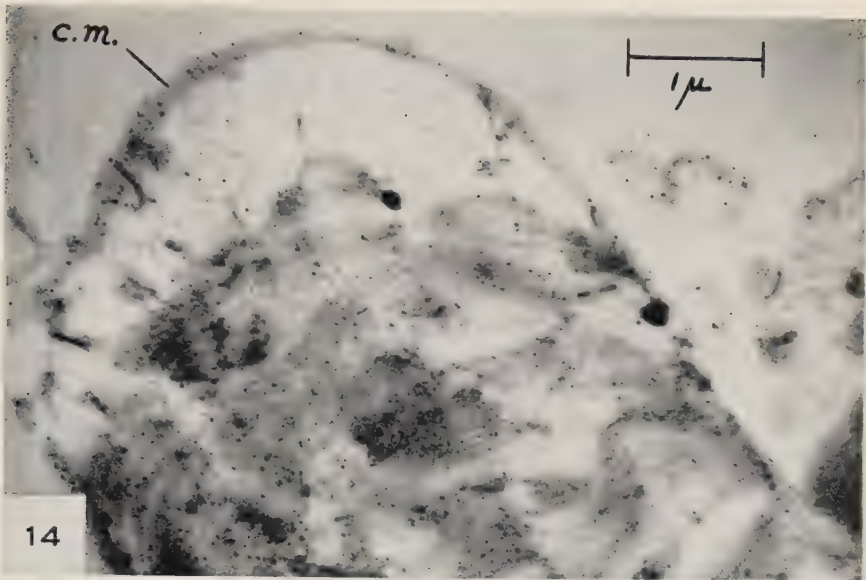
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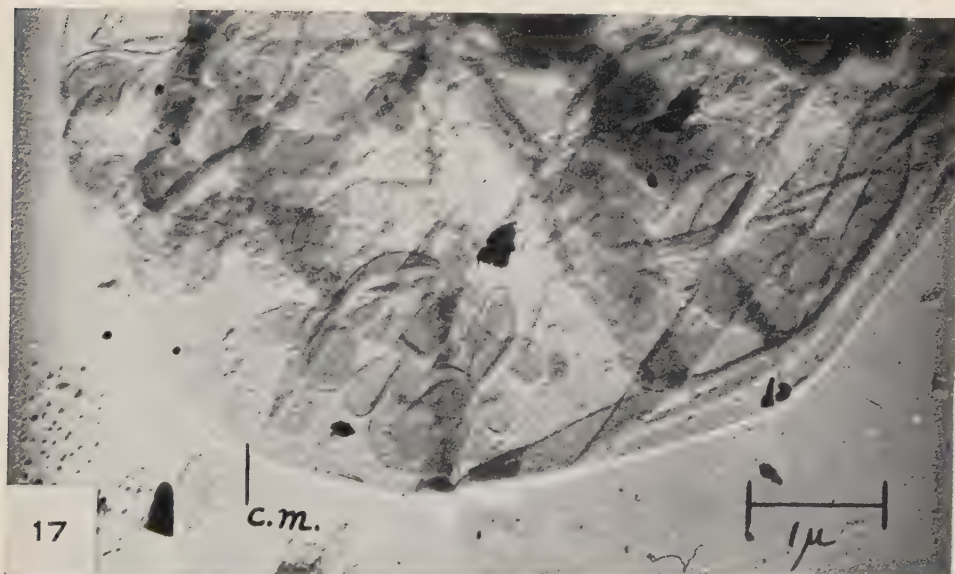
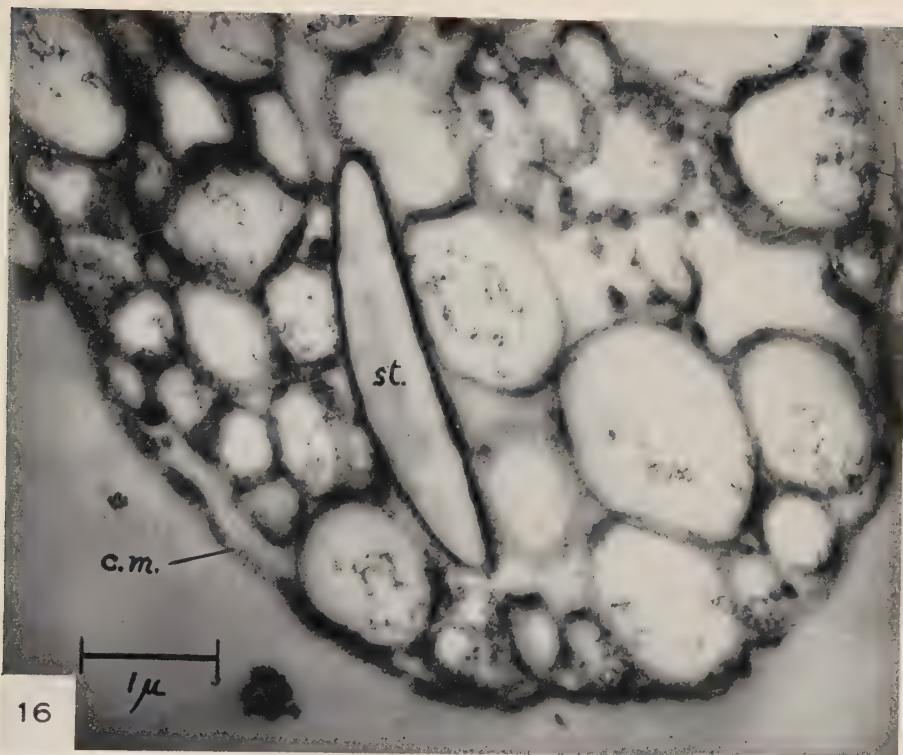
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EXPLANATION OF PLATES 1-8

chl., chloroplast or interlamellar material; *cl.*, chloroplast lamellae; *c.m.*, limiting membrane of chloroplast; *c.w.*, cell wall; *cyt.l.*, cytoplasmic lamellar structures; *m.*, mitochondrion; *st.*, starch granule; *st.v.*, starch vacuole; *t.*, tonoplast; *v.*, cytoplasmic vacuole.

PLATE 1

- Fig. 1.—Photomicrograph of chloroplasts *in vivo*, surface view. Starch grains present. The line represents 10 μ . Taken in oil immersion. $\times 4100$.
- Fig. 2.—Isolated chloroplasts viewed from above. Isolated in 0.5M glucose. $\times 4100$.
- Fig. 3.—Side view of isolated chloroplasts. Same medium as for Figure 2. $\times 4100$.

PLATE 2

- Fig. 4.—Cylindrical form of chloroplast isolated in distilled water, showing "vacuole" formation. The dark areas appeared green and the light ones almost transparent under the light microscope. The accompanying line represents 10 μ . $\times 4100$.
- Fig. 5.—Spherical bleb formation in chloroplasts isolated in distilled water. $\times 3600$.
- Fig. 6.—Chloroplasts isolated in distilled water showing conspicuous blebs at the ends and the green coloured reticulum between. $\times 4100$.

PLATE 3

- Fig. 7.—Transverse section of osmium-fixed cell of *Nitella*, showing the cell wall and two overlapping chloroplasts lying in the thin cytoplasmic layer. Note the chloroplast membrane, general lamellae organization of the chloroplasts, the starch vacuole, and the dense staining round bodies situated between bands of lamellae. Electron micrograph, $\times 29,500$.

PLATE 4

- Fig. 8.—Enlargement of upper left portion of Figure 7 to illustrate more clearly the stacks of lamellae enclosed by a limiting membrane. Note also the tonoplast. Electron micrograph, $\times 94,000$.
- Fig. 9.—Portion of a chloroplast *in situ*, showing clearly the well-ordered lipoprotein lamellae and the interlamellar material or chloroplast within the chloroplast membrane. Note tonoplast and lamellar structures in the cytoplasm. Electron micrograph, $\times 100,000$.
- Fig. 10.—Field similar to that of Figure 9, but showing more clearly the tonoplast and the cytoplasmic lamellar structures. Electron micrograph, $\times 120,000$.

PLATE 5

- Fig. 11.—Transverse section illustrating the orderly stacking of the lamellae in groups or bands, the intervening spaces being filled with interlamellar material or chloroplast. Electron micrograph, $\times 120,000$.
- Fig. 12.—Transverse section of cell kept in darkness. Note the thin dense membrane of the starch vacuole. Two closely applied chloroplast membranes run diagonally across the bottom left of the field. Electron micrograph, $\times 63,000$.

PLATE 6

- Fig. 13.—General view of the cytoplasmic layer in transverse section, including parts of two overlapping chloroplasts, several mitochondria, and a number of cytoplasmic vacuoles. Note the two dense spherical bodies within the chloroplast (lower left), the cytoplasmic lamellar structures (presumably elements of the "endoplasmic reticulum"), and the tonoplast closely applied to the cytoplasmic vacuolar membranes. Electron micrograph, $\times 80,000$.

PLATE 7

- Fig. 14.—Thin section of a swollen isolated chloroplast similar to those in Plate 2, Figure 4, showing cylindrical form with end bleb formation. This bleb involves only the intact chloroplast membrane. The chloroplast lamellae are still relatively well ordered, but much more widely separated than in the intact plastid. Electron micrograph, $\times 18,000$.
- Fig. 15.—Thin section of swollen isolated chloroplast similar to that in Figure 14, but showing much vacuolization between bands of lamellae. Electron micrograph, $\times 17,000$.

PLATE 8

- Fig. 16.—Thin section of a greatly swollen isolated chloroplast. The intact chloroplast membrane encloses a number of thin-walled vacuoles formed by fusion of the individual lamellae. Vacuolization is complete at this stage of swelling. The starch granule is apparently enclosed in a thin dense-staining membrane. The vacuoles contain reticular material, presumably the remains of the chloroplast. Electron micrograph, $\times 18,000$.
- Fig. 17.—Swollen chloroplast similar to that in Figure 16, but mounted by drying on a collodion film, followed by shadow-casting with uranium; ratio of shadow length to object height, 4:1. The intact chloroplast membrane is flattened on to the supporting film and can be seen to contain a number of similarly flattened vacuoles. Electron micrograph, $\times 18,000$.

PROTOPLASMIC STREAMING IN *TRADESCANTIA*

I. THE EFFECTS OF INDOLEACETIC ACID AND OTHER GROWTH-PROMOTING SUBSTANCES ON STREAMING

By JUDITH M. KELSO* and J. S. TURNER*

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Summary

The rate of protoplasmic streaming in the staminal hair-cells of *Tradescantia virginiana* is not affected by change of oxygen concentration in a hanging drop enclosing the hairs over the range 1.5-100 per cent. of oxygen in the surrounding gas phase. This allows experiments to be carried out without continuous renewal of the water drop.

Over the physiological range of concentrations, indoleacetic acid (IAA), indolebutyric acid (IBA), naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) all modify the rate of protoplasmic streaming in these cells.

These growth-promoting substances, added alone, bring about changes in the rate of streaming within 10 min; maximum effects are reached in 30 min, and the rates return to the normal in 60-70 min (exceptions to this rule are noted for NAA (high concentrations) and 2,4-D (low concentrations)).

Low concentrations of the auxins stimulate the streaming, high concentrations depress the rate, and intermediate concentrations are without effect. The auxins thus affect streaming much as they do the growth of stems, and the optimal concentrations for stimulation are similar for both processes. The "total effect" (T.E.) for each growth substance may be estimated as the total extra or diminished distance travelled by a protoplasmic particle in the presence of the applied substance. The maximum positive T.E. is given at 1 mg/l (1 p.p.m.) for IAA and IBA, and at 5 mg/l for NAA.

There is a marked pH effect for the reaction of streaming to IAA: increase in pH renders the applied solution much less effective over the whole range of concentration.

The temporary effects of indoleacetic acid on streaming, both stimulation and inhibition, are stabilized, near their maximal values, if fructose or malic acid are added with the auxin.

These results largely confirm those obtained by Thimann and Sweeney for the *Avena* coleoptile, except that the concentration for IAA giving maximal T.E. for *Avena* is as low as 0.01 mg/l. Moreover, for *Avena*, malic acid not only stabilizes the auxin effect but alters the threshold of response.

An improved apparatus is described for measuring the rate of protoplasmic streaming in cells.

I. INTRODUCTION

In 1937 Thimann and Sweeney reported a marked effect of indole-3-acetic acid (IAA) and other growth-promoting substances on the rate of protoplasmic streaming in the cells of the *Avena* coleoptile. Extension of this work (Sweeney

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and Thimann 1938, 1942; Sweeney 1944) led the authors to put forward a hypothesis that auxin effects on streaming, growth, and respiration are all related to the action of auxins on a 4-carbon respiratory cycle. Their results were questioned by Olsen and duBuy (1940), who failed to obtain stimulation of streaming with IAA, although, with higher concentrations, they obtained slight inhibition; they argued that the effects described by Thimann and Sweeney were due to impurities in the auxin. Sweeney (1941), however, attributed these negative findings of Olsen and duBuy to their use of coleoptile sections infiltrated with water; she found that under such conditions no acceleration of streaming was produced by IAA. While not adding further data on the effect of IAA on streaming, Showacre and duBuy (1947) countered Sweeney's argument indirectly by showing that infiltration of *Avena* coleoptile sections does not prevent the acceleration of growth by IAA. The matter appears to have rested here, and Audus (1949), on reviewing this field, remarked that "the experiments of Sweeney and Thimann should be repeated under the experimental conditions described in their papers." In 1947 we began to study this problem in another way, by the choice of a different species in which the complications due to the use of cells organized in tissues could be avoided. After some preliminary work with fungi, algae, and flowering plants, we selected the mature staminal hair-cells of *Tradescantia* as suitable material. The results presented in this paper are in accord with most of those obtained by Sweeney and Thimann, and will form the background for later papers on the effects of respiratory inhibitors on streaming, and on a marked effect of oxygen which has been demonstrated for the reaction of streaming to IAA.

II. METHODS

Streaming was studied in single cells of the staminal hairs of *Tradescantia virginiana*. A few hairs were cut from stamens taken from open flowers, mounted in water (double-distilled in glass), or in the appropriate solutions on the surface of a circular coverslip $1\frac{1}{4}$ in. diameter, the ends of the hairs being kept in position with petroleum jelly. The coverslip was then inverted to form the top plate of a chamber made from a brass ring and glass slide. Gas could be passed through the chamber by means of two hypodermic needles sealed into the edges of the ring. The coverslips were sealed into position with petroleum jelly. All experiments were carried out in a constant-temperature room at 24°C , where the basal streaming rate showed relatively small fluctuation. The rate of streaming was not appreciably affected when the microscope lamp was switched on; nevertheless, the light was kept on continuously throughout an experiment.

During the first year of the experiments the streaming was observed through a monocular microscope at a magnification of $\times 625$, and a stop-watch was used to time the movement of the smaller particles in the cytoplasm. Readings were made at 5-min intervals, each reading being the mean of 10 individual measurements of the time taken for a single small particle to travel past 10 scale divisions of the eyepiece micrometer. Unless otherwise stated, the

chamber contained air, and gas was not passed through it during measurements. In all experiments streaming rate was determined in distilled water for 15-25 min, by which time a steady rate of streaming had been established. Good uniformity was observed in the basal streaming rate in water of hair-cells from the one flower, but in most experiments on the effect of a given factor one cell was used as its own control, its basal rate of streaming being determined before the conditions were changed. The distilled water was then drawn off from the hairs, with filter paper, and the test solution added. All solutions were made up in distilled water, and brought to 24°C before use. The pH values of the solutions used were determined by means of a glass electrode, and adjusted when necessary with hydrochloric acid or sodium hydroxide.

During and after the second year of the experiments a device was used which saved eyestrain and probably led to a smaller subjective error in determination of streaming rate. However, the results obtained with this apparatus were not different from those obtained earlier using direct ocular measurements.

The apparatus is based upon that described by Sweeney and Thimann (1942). In their apparatus a moving bead belt is viewed at stage level with the streaming protoplasm, via a camera lucida. The bead belt is driven by a constant-speed motor and speed variations of 1-2 are obtained by altering the tension of the driving belt. Because we considered that it might be difficult to reproduce conditions accurately over a long period, with such a method of varying the speed, the following apparatus was devised and constructed for us by Mr. Matthaei of this Department. The principle applied is that the apparent speed of a given object varies with the viewing distance. The apparatus is illustrated in Plate 1. A small synchronous motor drives a disk at 1 r.p.m. and a fog-developed strip of 35-mm film is fixed to the periphery of the disk (5). A pilot lamp illuminates the perforations of the film from behind. A slit on the shield (4) permits 10 perforations to be seen. The image of this slit and the moving band of perforations of the film are superimposed on that of the specimen through a camera lucida (2) and mirror (3). The appearance of the streaming protoplasm (9) and of the film image is shown in the photomicrograph (Plate 1, inset). The apparent speed of the perforation images is altered every few minutes until it coincides with that of the main stream of protoplasmic particles. Streaming was observed with a 3-mm objective under a total magnification of $\times 525$. Screen (4) with its accompanying motor and illuminated strip can be moved along the optical bench (6) by means of the endless belt (7). A pointer attached to (4) moves along a permanent fixed scale on the bench. The apparent streaming rate of the film perforation images is dependent on the distance of the film from the microscope. The instrument is calibrated from the formula

$$V^1 = V \frac{d}{mD},$$

where V^1 = velocity of the (matched) stream in mm/min,

V = surface speed of film drum (5) in mm/min,

d = 25 cm (unit distance of magnification),

m = magnification of microscope measured 25 cm from the exit pupil of microscope, and

D = distance between exit pupil of microscope and film drum.

The resulting speeds for various positions are marked off on a bench ruler in mm/min, and the scale extends from 0.51 to 0.14, giving a speed variation of about 1-3½.

The apparatus was tested by setting the pointer to a given speed after calibration according to the above formula, and then measuring the speed of a single moving light spot by direct observation and stop-watch. The two figures so obtained usually differed by less than 4 per cent.; occasional large discrepancies must be ascribed to personal errors in the direct method.

Critical illumination of the microscope was necessary for obtaining a clear contrasted image of the protoplasmic stream. A microscope lamp was used as the illuminant, with diaphragm adjusted according to the Köhler principle. Some difficulties were experienced in projecting the field stop of the lamp into the plane of the specimen chamber. This was overcome by removing the front lens of the condenser. The resultant numerical aperture of about 0.4 was enough for a good definition. A green filter was used in order to give clearer definition of the moving protoplasm.

The IAA (β -indolylacetic acid, BDH) was purchased at frequent intervals, only pure white crystalline material being used. The acid was dissolved in water, double distilled from glass at 20-25°C. Solutions were made up freshly for each set of experiments, or were kept between experiments in the dark at 0°C for not longer than 2 days; they were brought to the temperature of the constant-temperature laboratory (24°C) before use.

III. RESULTS

(a) *Basal Rates of Streaming in Water*

Flowers, picked when freshly opened, were placed with their stalks in water and kept in a cool place. Such flowers were used, for a period not exceeding 8 hr, as the source of supply of fresh hair-cells. Occasionally the apparently healthy hair-cells of freshly opened flowers showed no cyclosis. These cells were discarded, as in these experiments we were not concerned with factors initiating streaming.

Generally the protoplasm of the hair-cells was found to be actively streaming. The basal rate in a given hair-cell, mounted in water over air, was measured either directly by observation of single particles, or (later) by means of the apparatus already described.

In the first year, when direct timing of particles was used, the rates obtained were some 30 per cent. lower than those found for comparable periods in later years, by matching the speed of the stream of protoplasm. It is impossible now to determine whether this was due to physiological differences between the plants of the first and later years, or to the earlier choice of the rather

larger and slower particles for measurement (see Sweeney and Thimann 1942). It is, however, significant that, over a 2-yr period, the basal rate, as measured by both methods, showed a marked rhythm. It was at its minimum in July (midwinter) and showed two pronounced maxima, one in autumn (April-May), and one in spring (October-November). The maximum rate (using the apparatus) was approx. $7 \mu/\text{sec}$ and the minimum $6 \mu/\text{sec}$. Basal rates given by Sweeney and Thimann (1942) for *Avena* coleoptiles are between 12 and $15 \mu/\text{sec}$. They also found a seasonal variation in the basal rate, with a maximum in late summer.

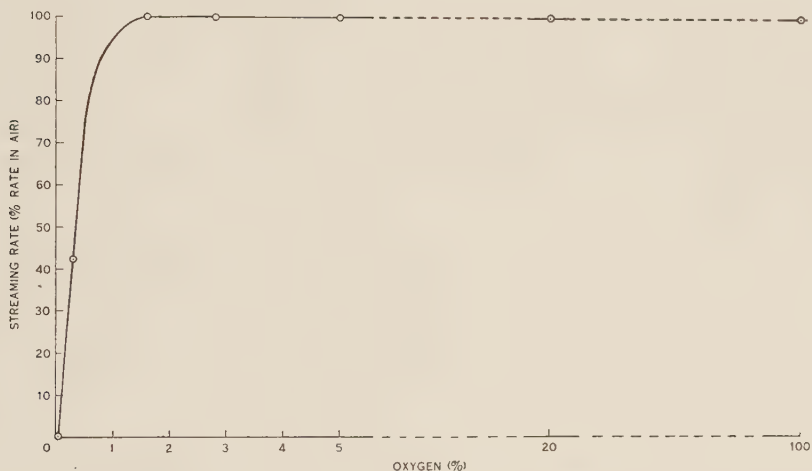


Fig. 1.—Relationship between oxygen concentration surrounding the hanging drop and the rate of protoplasmic streaming in the staminal hair-cells of *Tradescantia virginianum*. Each point on the curve is the mean of five separate determinations of the steady state rate, each expressed as a percentage of the rate in air.

(b) Streaming as Affected by the Concentration of Oxygen

Streaming in *Tradescantia* staminal hair-cells continues under conditions of low oxygen tension. Ewart (1903) reported that some workers were unable to stop streaming even by placing the hairs in pure hydrogen. However, in his own work with this species, streaming ceased in hydrogen within 15 min to 3 hr, the time varying with the temperature and with the age and condition of the cells. In our experiments, gases, including air, cylinder oxygen, cylinder hydrogen, and various mixtures of oxygen and nitrogen were passed through the experimental chamber and their effect upon the rate of streaming measured. The concentration of oxygen in each gas was determined in the Haldane gas analysis apparatus. The gases were bubbled through water before passing through the cell in order to prevent evaporation in the chamber. The results are graphed in Figure 1, similar results being obtained over 2 yr with various specimens of staminal hairs and using both ocular timing of particles and the apparatus described above. In all experiments the rate was first determined

in air and the change in rate, if any, measured when air was replaced by a different gas mixture. The new rate was measured for a period of at least 30 min, following the establishment of steady state, and rapid recovery to normal rate took place on return to air. All rates were expressed as percentages of the rate in air. It is clear that above about 1.5 per cent. oxygen (the critical value) the concentration of oxygen in the gas over the hanging drop has no influence upon the rate of streaming. Only when the concentration of oxygen was reduced below 1.5 per cent. was the rate of streaming reduced. In these cases the rate decreased immediately and reached a new steady state 15-20 min after the new gas was first introduced into the chamber.

A stream of hydrogen containing only 0.3 per cent. oxygen brought about a complete stoppage of protoplasmic streaming within 15 min. Recovery occurred almost at once when the current of hydrogen was discontinued, presumably owing to slight leakage of oxygen into the chamber, or to traces of oxygen in the water film. When cylinder hydrogen was again passed through the cell, streaming stopped within 3 min. Recovery was more gradual after prolonged passage of hydrogen.

The combination of the low critical value of oxygen for *Tradescantia* and the use of microscopic samples in hanging drops allows experiment in small closed vessels in which the water drop is in contact with air. In the work with *Avena* it had been found necessary to use specially aerated water for the longer experiments or to replace the liquid drop every few minutes or even continuously. If half a young coleoptile 4.5 cm long, split longitudinally, is placed under a coverslip, the resulting oxygen deficiency (due to respiration) leads to a decrease of streaming within a few minutes. The critical value of oxygen for *Avena* has not been clearly established; the results of Bottelier (1935) indicate that for young coleoptiles (grown for 96 hr at 26°C) the streaming rate is increased on passage from water charged with air to that saturated with oxygen. If, however, the critical value for the cell in *Avena* is low, the use of comparatively large (respiring) samples of tissue in each experiment must be responsible for the rapid decrease in streaming in unaerated or unchanged water drops.

(c) Effect of IAA on Streaming

In all the experiments described in this section the experimental chamber contained air. The rates of streaming for a given cell were first measured while the cell was in glass-distilled water, and this was then replaced, without change of temperature, by a drop of freshly made up IAA solution (measured pH 4.5-5, depending on the concentration). Rate measurements were made 3 min after the change and subsequently at 5-min intervals. In all, some 100 individual experiments of this kind have been made, and, with a few exceptions to be mentioned below, the results were consistent. Over the concentration ranges 0.1-10 mg/l and from 50-100 mg/l the IAA caused marked changes in the rate of streaming, these changes becoming apparent within 10 min of treatment. Figure 2 shows the results for a typical series of experiments with staminal hairs taken from the one flower.

(i) *Concentration Range*

(1) *IAA 0.1-10 mg/l: stimulation.*—These solutions all caused a transient increase in the rate of streaming, apparent within 3 min. Maximum rates, for each concentration, were reached within 20 min, and in all experiments the rates returned to the normal (i.e. to the rate in water) within 60-70 min. At the lowest effective concentrations (0.1 mg/l) the effect was small and simple,

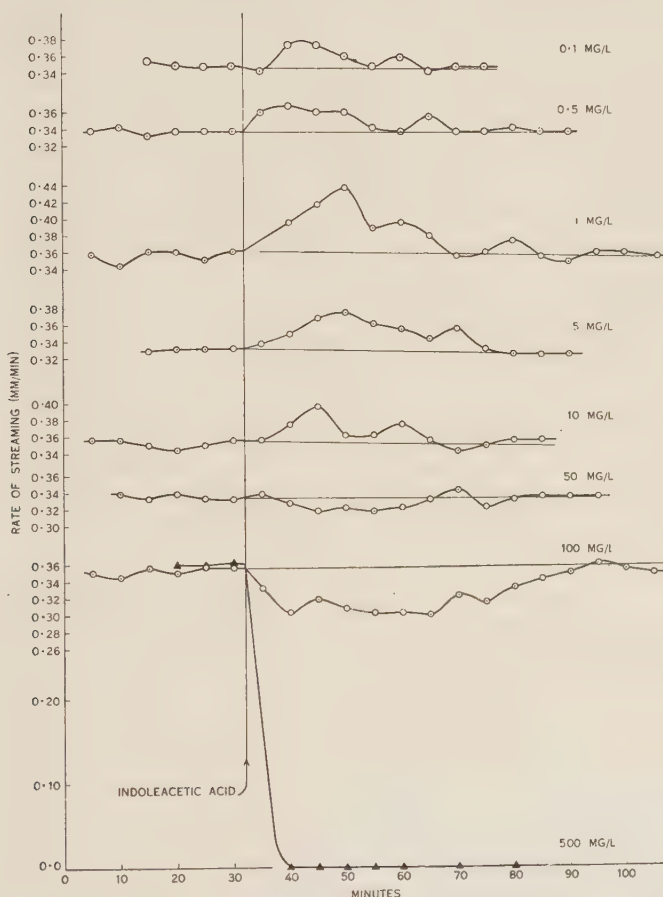


Fig. 2.—Effect of IAA solution, at various concentrations, on the rate of protoplasmic streaming. Each curve is obtained from measurements on a single hair-cell, all hair-cells from the same flower.

and it was over within 25 min. With concentrations near 1 mg/l the effect was greater and more prolonged. The curves often showed a curious double effect, a second brief stimulation interrupting the fall to the normal rate. The maximum increase was of the order of 20 per cent. of the basal rate and was given by the concentration of 1 mg/l. Effects given by 10 mg/l closely resembled those given by 0.1 mg/l, and concentrations between 10 and 50 mg/l had little effect on the rate.

(2) IAA 50-100 mg/l: *inhibition*.—These solutions caused an inhibition of streaming, which was not complete and not lasting. The time curves are in the form of mirror images of those described in section (1). The effects were

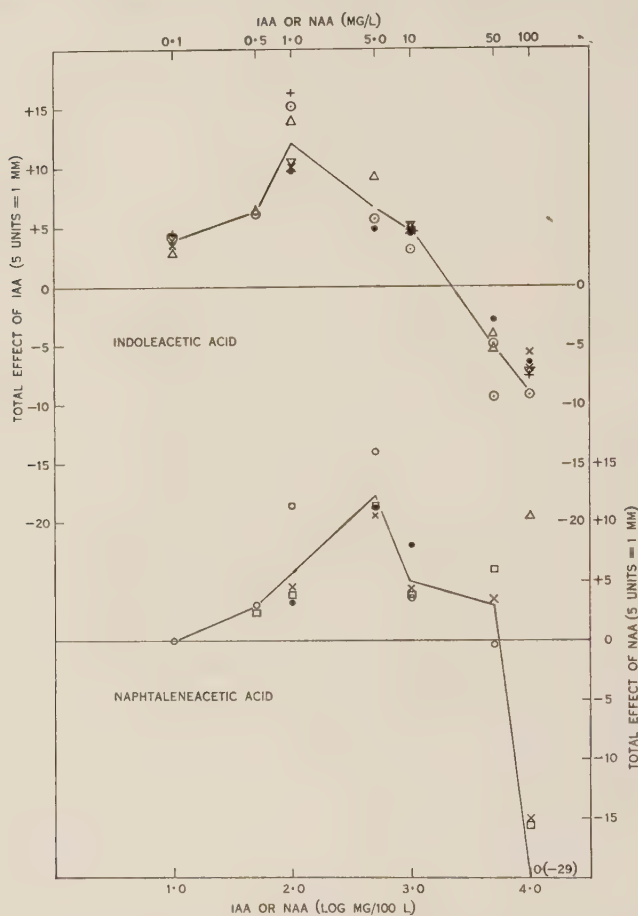


Fig. 3.—(Above) *Total effects* of various concentrations of IAA on the protoplasmic streaming. The lines join the means of points obtained in five separate series of experiments with different flowers. Each point represents the area under the rate/time curve (Fig. 2), using the extrapolated rate in water as the base line. (Below) The same, for α -naphthaleneacetic acid. The lines join the means for three separate experiments (see Fig. 6).

also apparent within a few minutes, maximal inhibition was reached within 20 min, and the rate again returned to the normal rate within 70 min. The greater the concentration, the greater was the inhibition.

(3) IAA > 100 mg/l.—Here the effect was a progressive inhibition, not followed by recovery, and eventually leading to a complete stoppage of all streaming.

These results show a striking parallelism with those obtained by Thimann and Sweeney (1937) with the *Avena* coleoptile. The general form of the curves is the same and the time scales are of the same order, recovery from auxin effects being somewhat slower for *Tradescantia*, and the stimulatory effect on the rate being smaller than demonstrated for *Avena*. The major difference is in the concentrations of auxin necessary to bring about similar effects in the two tissues. This quantitative difference is best shown if we follow Thimann and Sweeney and express the *total effect* of a given auxin concentration as the area under the curves of rate/time, using the rate in water as the base line. This is of value as giving a figure for the total extra or diminished distance (5 units = 1 mm) travelled by a protoplasmic particle when in the presence of applied auxin, and it is particularly useful in our experiments where a double-headed effect is given by the lower concentrations, making other comparisons difficult. In Figure 3 we plot the results obtained with five series of experiments, in each of which hairs from the same flower were tested with a range of concentrations of IAA. The values for the total effects are plotted against the concentration of IAA on a logarithmic scale, the means for the total effects at each concentration being joined by straight lines. The result is closely similar to that already described for the *Avena* coleoptile by Thimann and Sweeney, a major difference being that the curve for *Tradescantia* is shifted bodily to the right, the maximum effect of auxins being given by concentration of 1 mg/l, whereas for *Avena* the maximum effect is given by 0.01 mg/l. The results for *Tradescantia* are much more closely similar to those obtained by Thimann and Sweeney for the elongation of *Avena* coleoptiles plotted against the logarithm of the auxin concentration. The comparison is made clearer in the generalized summary of some auxin effects given in Figure 4.

In making these comparisons it may be pointed out that the growth tests take 24 hr, while the streaming effects subside within 1 hr; the *Tradescantia* hair-cells are fully grown and borne exposed to air on flowers, while the *Avena* cells are those of a rapidly growing organ with some cut surfaces. The curve for *Avena* streaming is shifted still further to the left if younger coleoptiles are used (Thimann). It is clear that the streaming of protoplasm in *Tradescantia* is markedly affected by IAA over a range of concentrations which is known to be of physiological importance in the growth of plant shoots.

The results described above were obtained consistently in experiments carried out during the first 2 years of the investigation. It seemed therefore that one might be able to use *Tradescantia* as a test material for the determination of auxin concentrations. Further work showed that this would be a possibility only if the *Tradescantia* plants were grown under standardized concentrations. In the great majority of our experiments the curve of Figure 3 applied, and it was often possible (for one of us) to identify an unknown IAA solution (e.g. to state whether its concentration was of the order of 0.1, 1.0, 10.0, or 100 mg/l) by using the streaming test. However, from time to time we have found that staminal hairs taken from apparently normal flowers react abnormally in the streaming test, being much less sensitive than normally. In such hairs inhibition of streaming was not given even by concentrations of 500 mg/l; they were

not used for the experiments under consideration. So far we have not sufficient data to enable an attempt to correlate these abnormalities with climatic data; these abnormal results were obtained in about 5 per cent. of the experiments, and with hairs taken from plants flowering at the end of the season or in greenhouses given supplementary illumination.

(d) *Hydrogen Ion Concentration and the Auxin Effect*

In most experiments we followed Thimann and Sweeney in adding IAA without additional buffer at a pH which varied between 4.0 (100 mg/l) and 5.5 (0.1 mg/l). As the distilled water of the control experiments was acid, not being free from CO_2 , the change in acidity due to the auxin was not large.

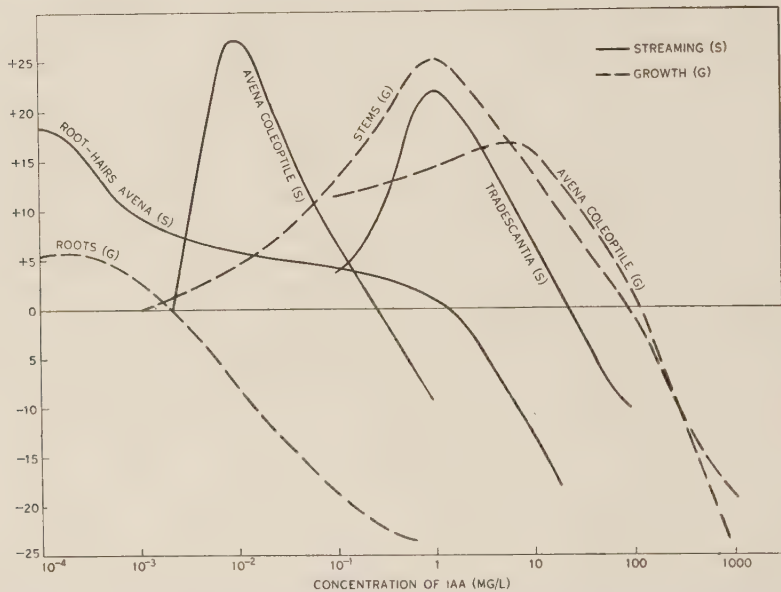


Fig. 4.—A generalized scheme showing the concentration ranges over which IAA exerts its effects, in different plant organs, on growth and on protoplasmic streaming. Data from Thimann and Sweeney (1937), Sweeney (1944), Audus (1953), and present work. Ordinates, increase or decrease in elongation or in rate of streaming; the scale for the ordinates is arbitrary, and comparisons of percentage effects are not attempted.

Two questions arise, however:

- (1) Is the "auxin effect" merely due to pH change?
- (2) Does the pH of the external solution markedly change the response to auxin?

(1) In our experiments change of pH in the external solution, in the absence of auxin, did not change the rate of streaming. Thus, with *Tradescantia* we found no change in the rate of streaming on passage of the hair-cells from water to phosphate buffers at pH 4 and pH 7. Thimann and Sweeney (1937) showed that, with *Avena*, acetic acid at concentrations between 0.001 and

0.000001M had no effect on streaming, while Sweeney stated that the addition of alkali to give a pH of 9 also did not affect the streaming rate in this plant. Becker (1936), working with *Tradescantia*, reported that there was no effect on streaming on the addition of various acids, including mineral acids and weak organic acids at pH 3.8. Lepow (1938) reported that the rate of streaming in *Physarum* was not altered by change of pH between 5 and 9. Like Thimann and Sweeney, we have found that the IAA effect is also given by certain other plant hormones, whereas malic acid and succinic acid had no effect on the rate of streaming when added alone at low pH. In spite, therefore, of a few records of a direct effect of pH on protoplasmic streaming (Seifriz 1943, mostly with green cells), we are of the opinion that our results must be explained in terms of the auxin and not of pH change *per se*.

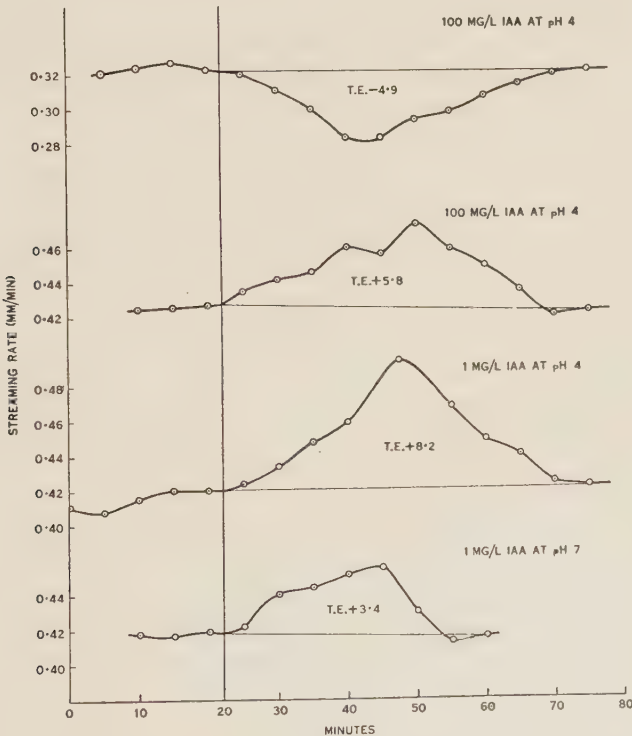


Fig. 5.—Effect of IAA on protoplasmic streaming, as modified by the pH of the solution. T.E. = total effects, the area under the curve with the control extrapolated as the base line.

On the other hand, we are in agreement with the Thimann school that the pH of the auxin solution is of great importance. A thorough investigation of this aspect has not so far been attempted but enough work has been done to show that, on changing the pH of the auxin solution from 4 to 7, there is a decrease in the effective concentration of the auxin. Thus (Fig. 5) a high concentration of auxin (100 mg/l, pH 4) caused an initial fall in the streaming

rate, followed by a rise to the normal, with a total effect of -4.9 . The same concentration applied at pH 7 brought about a temporary stimulation in the streaming (total effect $+5.8$) similar to that given by a weaker auxin solution at pH 4. Similarly, a low concentration of auxin (1 mg/l) applied at pH 4 caused a marked transitory stimulus in the rate (total effect $+8.2$), whereas the same concentration at pH 7 brought about a smaller increase (total effect $+3.4$). Thus, although we have not enough data to plot the complete curve, there are strong indications that if we plotted total effect against log concentration of auxin at pH 7, a curve similar to that of Figure 3 would be obtained, except that it would be shifted bodily to the right by one unit of log concentration. Thimann and Schneider (1938) obtained a closely similar result for streaming (but not for growth) with the *Avena* coleoptile, and explained it as being due simply to the effect of pH on the dissociation of the auxin,—“at pH 7, when auxin consists of 1% acid and 99% salt, auxin action (on streaming) is as though its concentration has been reduced to 1/100 of its concentration at pH 4.” This would imply that the pH effect is one largely concerned with the rate of entrance of the acid molecule into the protoplasm, and this hypothesis would also serve to explain the lack of a pH effect on growth, the measurement of which takes much more time than does measurement of change in streaming rate. However, it must be noted that while increase in pH (like simple dilution of an acid solution) changes the *total effect* of the auxin, it does not materially alter the rate at which the streaming reacts to the applied solution (Figs. 5, 2). This does not lend support to the explanation offered, which is probably an over-simplification of a very complex situation (see Hanly, Rowan, and Turner 1952). Reinhold (1954) has suggested that for one tissue, at least, the uptake of IAA probably involves both physical and metabolic processes, and while pH mainly affects a physical process, the fall in the rate of uptake with increasing pH is not parallel to the fall in the mean concentration of undissociated IAA molecules in the medium.

As stated above, we followed Thimann and Sweeney in using the weakly buffered pure IAA solutions in our experiments, and it seems probable that for both investigations the form of the curves obtained at the lower concentrations would be changed if all experiments had been done in phosphate buffer of pH 4 instead of in water at pH rising slightly with dilution. However, this change is likely to be small, and cannot affect the general interpretation of the results.

(e) Effects of Auxins other than IAA

Thimann and Sweeney (1937) showed for *Avena* coleoptiles that the growth-promoting substances IAA, coumaryl-3-acetic, and *allocinnamic* acids all affect streaming in a similar way; quantitatively their effects are what would be expected from their activity as auxins. We have compared the effects of IAA on *Tradescantia* streaming with those produced by indolebutyric acid, indoleacetonitrile, α -naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid.

The relative activity of indolebutyric acid, determined by four different growth tests (Thimann and Schneider 1939), is between 8 and 190 per cent.

of that of IAA. Its effect on streaming in *Tradescantia*, like its effect on the straight growth of *Pisum*, is quantitatively closely similar to that of IAA itself. Maximal temporary stimulation was given by a concentration of 1 mg/l; a solution at 10 mg/l stimulated very slightly, and one at 100 mg/l caused a temporary depression in the rate of streaming.

A few tests with indoleacetonitrile, which is more active than IAA in promoting cell elongation (Bentley and Bickle 1952), showed that it too modifies the streaming rate; a concentration of 10 mg/l stimulated streaming, and concentrations between 50 and 100 mg/l inhibited it. There were, however, indications that these effects were more permanent than those brought about by IAA itself, and further work with this substance is envisaged.

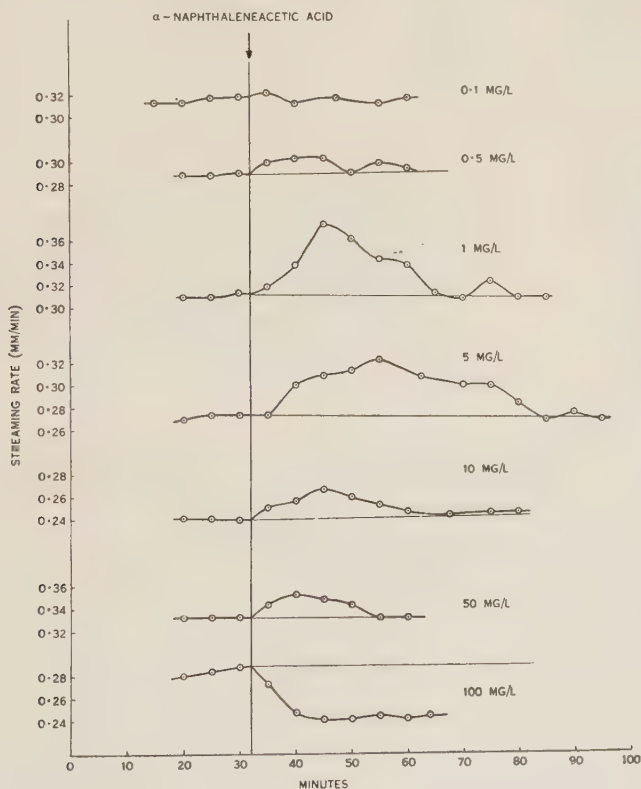


Fig. 6.—Effect of α -naphthaleneacetic acid solution at various concentrations on the rate of protoplasmic streaming in *Tradescantia*.

For α -naphthaleneacetic acid a more complete investigation has been carried out. This acid is reported as having between 2.5 and 23 per cent. of the growth-promoting activity of IAA (Thimann and Schneider 1939), except for the slit-stem *Pisum* test, in which it is nearly four times as active. Against streaming, in *Tradescantia*, it acts in a similar manner to IAA, with two qualifications. At concentrations between 0.1 and 50 mg/l it temporarily stimulates

streaming (Fig. 6); in the presence of the applied acid the rate returns to that of the water control within 40-50 min. The optimal concentration, however, was 5 mg/l, which caused a 20 per cent. stimulation in the rate. The "total effect" for three separate series of experiments is plotted against concentration in Figure 3, and comparison shows that *a*-naphthaleneacetic acid is less active than IAA in its effect on streaming. Another feature of difference so far found between these auxins, however, is that the partial inhibition of streaming brought about by concentrations of 100 mg/l is temporary for IAA and more permanent for NAA (Fig. 6). For this latter the inhibition reaches 70 per cent. within about 15 min; the new rate is maintained for a further 20-25 min, and then falls slowly to zero. We have not, with this acid, found recovery from inhibition such as is characteristic for the corresponding concentrations of IAA.

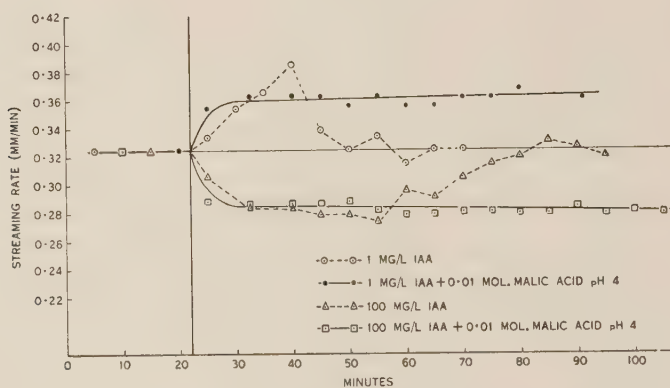


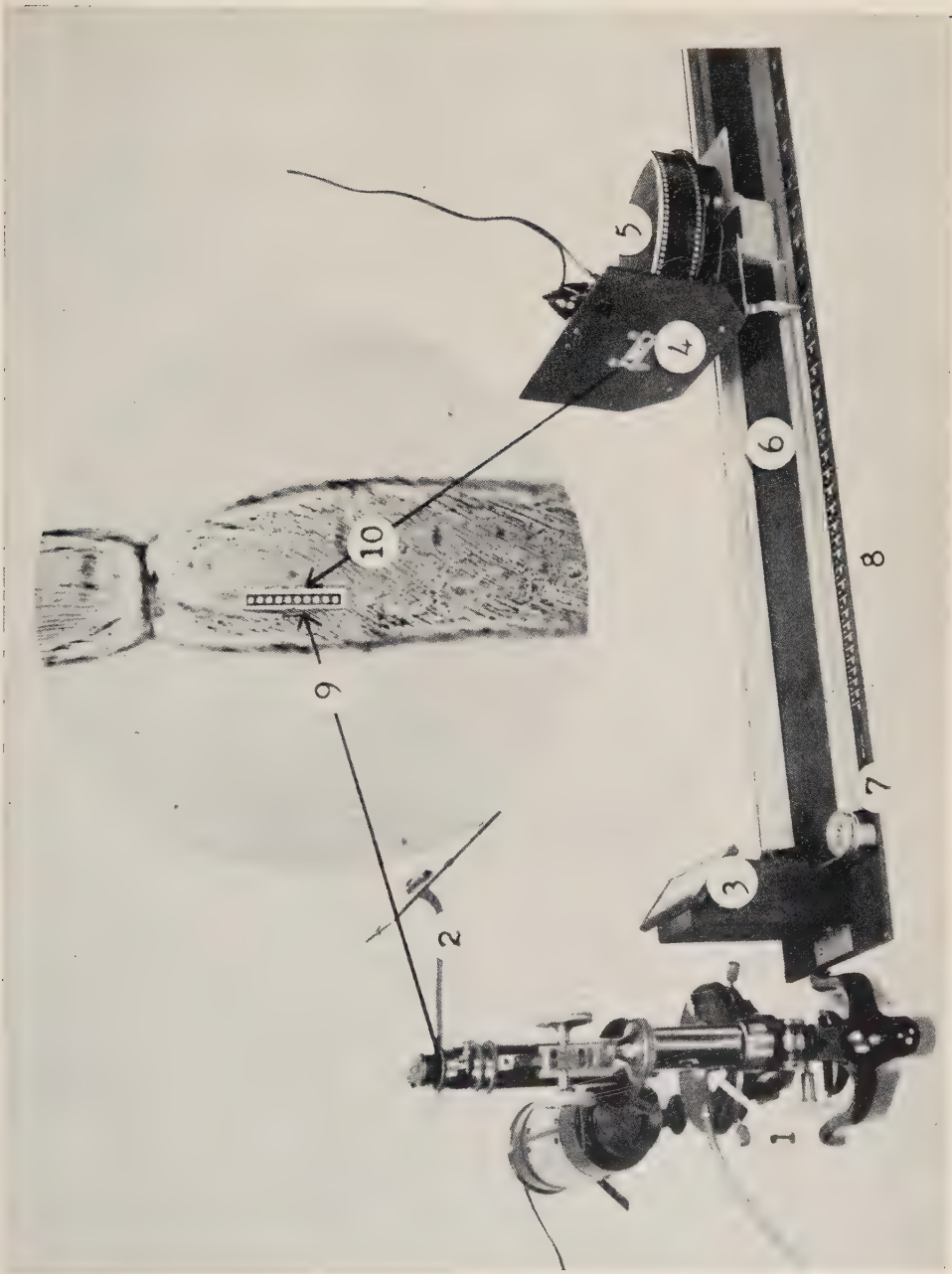
Fig. 7.—Effect of IAA on protoplasmic streaming as stabilized by the addition of malic acid (0.01M) with the auxin.

A few preliminary experiments with 2,4-dichlorophenoxyacetic acid show that this substance affects the rate of streaming, but its action is again different. A concentration of 100 mg/l caused a marked but only temporary depression in streaming rate, the effect being very similar to that due to the same concentration of IAA. Lower concentrations, between 0.5 and 50 mg/l of 2,4-D, brought about slight and apparently permanent stimulation of streaming, the maximum effect (10 per cent., due to 10 mg/l) being reached gradually over a period of 40 min. These stimulation effects, although smaller, resemble those obtained with IAA in the presence of sugar or malic acid.

(f) The Role of Carbohydrate and Organic Acids

A striking feature of the auxin effects in both *Avena* and *Tradescantia* is that in the great majority of experiments the streaming rate returns to that of the water control within 1 hr. For *Avena* the period of stimulation or inhibition is about 30 min. For *Tradescantia*, in 53 experiments, it varied over the range 20-60 min but seemed to be independent of the concentration of auxin. The mean for all concentrations was 38 min (S.D. 10.6 min). It must be emphasized

PROTOPLASMIC STREAMING IN *TRADESCANTIA*



Photograph of apparatus used for measuring the rate of protoplasmic streaming; description in text. *Inset*, photomicrograph of *Tradescantia* hair-cell showing the appearance of the projected image of the band of film perforations.

that recovery from maximum stimulation (1 mg/l) took the same time as recovery from inhibition (100-200 mg/l).

Sweeney and Thimann (1938) obtained recovery from stimulation even although fresh auxin solutions were continually supplied; under such circumstances there was only a "second slight upward trend which suggested a return of the auxin effect" an hour after the first stimulation had subsided. Moreover, if, after the rate had returned to normal, the cells were washed with water, a second stimulation by auxin could be obtained if a period of 30 min in water was allowed to elapse, or, alternatively, if sugar were added with the auxin. They explained the transient nature of the auxin effect as due to the exhaustion of a substrate; and supporting this view is their observation that the initial auxin effects on streaming could be stabilized by adding carbohydrate or malic acid with the auxin.

For *Tradescantia* our findings are:

- (1) Fructose alone (0.03M) and malic acid alone (0.01-0.1M, pH 4) have no effect on the rate of streaming, at least during 1 hr after their addition.
- (2) When added either with IAA or indolebutyric acid at pH 4, fructose (0.03M) and malic acid (0.05M) modify the auxin effect on streaming in the same way. They stabilize the effect for each concentration of auxin, whether it be stimulation or inhibition. They do not alter the threshold of response, nor do they shift the position of the optimum, maximum stimulation still being given by 1 mg/l auxin. Malic acid, but not fructose, significantly increases the rate at which maximum stimulation is attained.* The stable rates are quickly reached and maintained for at least 1 hr; in one experiment with 0.1 mg/l auxin and 0.01M malic acid, for at least 2 hr. The percentage stimulation or inhibition reached in the presence of fructose or malic acid is of the same order as the maximum figure for the transient effects caused by the same concentrations of auxin added alone. Typical results are plotted in Figure 7.

The most effective concentration of malic acid in our experiments was between 0.01 and 0.1M. At 0.001M malic acid prevented the complete recovery from stimulation due to 1 mg/l auxin, but was not fully effective in stabilizing the response. Sweeney and Thimann used this concentration (0.001M) with *Avena* as it had an optimal effect on respiration. It lowered the threshold of response to auxin some 10 times; moreover, in long-soaked sections the response to auxin alone was small, the response in the presence of malic acid was normal. Such results have not so far been obtained with *Tradescantia*, for which, in

* Times required for maximum stimulation from addition of solution:

Auxin alone (53 experiments) 15.4 min; S.D. 7.1 min.

Auxin and fructose (4 experiments) 15.5 min; S.D. 6.5 min.

Auxin and malic acid (8 experiments) 4.4 min; S.D. 2.1 min.

Difference auxin acid and malic and auxin alone highly significant at 0.1 per cent. level.

this respect, fructose and malic acid are interchangeable. Moreover, the combined effects of auxin and fructose are not enhanced if malic acid is also added. Thus, a mixture of auxin (1 mg/l) with 0.5 per cent. fructose gave a steady enhanced rate of streaming; when this solution was replaced by one containing auxin (1 mg/l), 0.5 per cent. fructose, and 0.01M malic acid there was no change in the rate of streaming.

The recovery from the effects of auxin (added alone) cannot be due to the rapid removal of all auxin from the external solution. Several experiments have shown that the same drop of IAA solution will bring about similar streaming effects (e.g. stimulation followed by recovery) in successive experiments with two different hair-cells. This result is in agreement with that mentioned

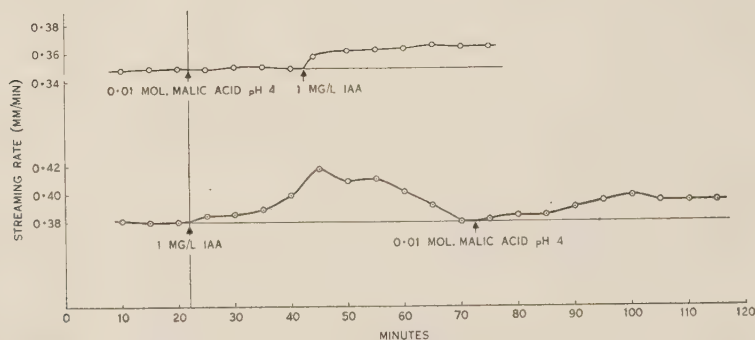


Fig. 8.—Effects on the rate of protoplasmic streaming of malic acid and IAA, added alone and consecutively. Description in text. At the first arrows the reagent added alone; at the second arrows the second reagent is added after the removal of the first.

above for *Avena*, in which recovery occurs even when the bathing solution is continuously renewed. Nor is recovery due to the destruction of auxin within the cell. In three separate experiments maximum stimulation was given when IAA was added alone at 1 mg/l, pH 4 (T.E. + 8.1, + 6.3, + 6.6); when the rate had returned to normal, after 50 min, the IAA solution was withdrawn, the cells momentarily washed with water, and then malic acid alone (0.01M, pH 4) was added (Fig. 8). In each experiment the streaming rate increased, rather slowly, to a new steady state. This was lower than would be expected for the concentration of auxin originally added, but these results suggest that after stimulation has subsided auxin is still present in the cell, and can exert its effect if malic acid is added. A somewhat similar experiment shows also that malic acid can pass into the protoplasm, without affecting its streaming rate, and become available to stabilize the effects of auxin added later. Thus (Fig. 8) when malic acid (0.01M, pH 4) was added alone there was no effect on streaming; when, however, after 20–25 min, the solution of acid was removed, the cells rinsed with water and mounted in IAA solution (1 mg/l), then a *stabilized* stimulation of streaming resulted.

One hypothesis, already mentioned, is that these results may be explained if the sugar and the malic acid are themselves the substrate (or provide the

substrate) for an auxin reaction. We have some evidence, however, which conflicts with this view, and full discussion of the cause of recovery from the varied effects of auxins on streaming will be left to a later publication.

The earlier findings of Thimann and Sweeney as to the effects of IAA and other growth-promoting substances on the rate of protoplasmic streaming have therefore been confirmed. It seems likely that further work in progress along these lines will add to our knowledge, not only of the nature of protoplasmic streaming, but also of the mode of action of auxins in the plant cell.

IV. ACKNOWLEDGMENTS

This work was carried out while the junior author (J.M.K.) was in receipt of a research scholarship in the University of Melbourne. We are most grateful to Mr. E. A. F. Matthaei, lecturer in Microscopy in this Department, for the design and construction of the apparatus described.

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CORRELATION BETWEEN THE ELECTRIC CURRENT GENERATED BY A BEAN ROOT GROWING IN WATER AND THE RATE OF ELONGATION OF THE ROOT

By B. I. H. SCOTT,* A. L. MCAULAY,* and PAULINE JEYES*

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Summary

Methods of measurement of the electric fields produced by plants have been developed which eliminate artefacts commonly present in such investigations.

The normal potential pattern in the water surrounding actively growing bean roots is described.

When rates of elongation of roots are controlled by two methods which produce very different types of overall metabolic change, the potential pattern is shown to be correlated with the rate of elongation.

Values are given of the electric power dissipated in water in which a bean root is growing, and of the current generated by the root in the water.

I. INTRODUCTION

Experiments made here and elsewhere show clearly that electric potentials measured on the surface of plant organs depend on a number of causes. In addition to those due to the plant itself when in its normal steady state of metabolism, there are a number of other sources of potential difference, some of them generated by the plant itself owing to treatment it receives during measurement, and others introduced from outside.

In this paper a new approach is made to the problem of the bioelectric phenomena associated with plant metabolism. The effect of the process of measurement on the observations made is entirely eliminated by measuring the potential fall down currents produced by the plant in an external medium.

When a bean root grows actively in aerated water it generates an electromotive force which passes current through the water. This paper describes measurements which have been made of the ohmic potential differences due to these currents in the water adjacent to the root. The pattern of these potentials is characteristic and reproducible when the root is growing strongly.

The paper further describes experiments in which the growth of the root has been controlled and in which a correlation has been found between the change in potential and the rate of elongation.

By addition of auxin of suitable concentration to the bath in which the root is grown, it is possible to arrest growth and observe the change in potential pattern. This process proves to be reversible, as removal of the auxin allows the plant to grow again and the potential pattern to recover.

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A similar correlation is observed when the temperature at which the roots are grown is raised, but the root cannot be restored to its original condition of normal growth by lowering the temperature.

II. SOME CAUSES OF THE CONFLICTING RESULTS OBTAINED IN PREVIOUS INVESTIGATIONS OF THE ELECTRIC FIELDS OF PLANTS

A number of investigations of the electric potential differences associated with plants have been made in the past. An examination of the literature shows very little agreement between the results obtained.

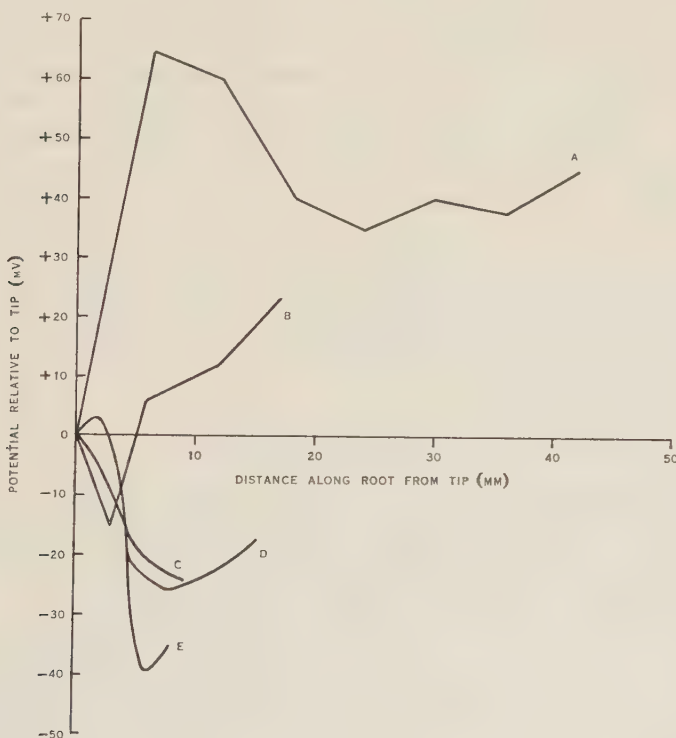


Fig. 1.—Comparison of the results obtained in several investigations of the relation between the external potential of the root relative to the tip and distance from tip along the root. Data obtained from A, Thomas (1939) (bean); B, Lundegårdh (1940) (wheat); C, McAulay, Ford, and Hope (1951) (maize); D, Lund and Kenyon (1927) (onion); and E, Ramshorn (1934) (bean).

Some of the results obtained using roots as material are compared in Figure 1, in which the potential of the root relative to the tip is plotted against the distance from the tip. It is evident that there is a wide diversity between the results of different observers. It is true that these results are not directly comparable since they have been obtained under a variety of conditions, and some

of the variation between them is certainly due to such factors as the type of plant used, its age, and the state of its environment. Nevertheless, a critical examination of the experimental techniques employed in the investigations shows that in most cases the measured potential differences were due not only to the plant e.m.f.'s but also to factors introduced by the measuring techniques. In particular, the following causes can be recognized as responsible for many of the variations in the results recorded.

The effect of variations in salt concentration at the point of contact has been almost invariably overlooked in previous investigations. Salt concentration changes are produced by drying and are due also to salt uptake by the plant. The resulting potential changes can be quite large, and are often greater than the plant potential it is desired to measure.

To illustrate the magnitude of the potentials set up in this way, it is found that if the concentration of KCl at one point of contact on a bean root is changed by a factor of 10 (the concentration at the other contact remaining unchanged) the potential difference between the contacts changes by more than 30 mV (Hope 1951).

Other factors which may obscure the true steady state electric pattern generated by the plant are variations in environment, either of the plant as a whole, or local variations at the point of contact. These may include temperature, humidity, salt concentration, the amount of aeration, and light and gravitational influences. In addition, stimulation or injury by the measuring probes can produce large potential differences.

III. EXPERIMENTAL METHODS

Most of the sources of error listed above are liable to occur if the measuring probes are in mechanical contact with the plant. To avoid this, three methods have been developed and used to measure plant potentials without making mechanical contact with the surface of the plant.

Two of these methods are suitable for measuring electric potentials of plant surfaces in air, while the other is suitable for use with plants grown in water. In the first, which has been described elsewhere (Blüh and Scott 1950), a probe is set into vibration close to the plant, and its potential is adjusted until there is no field between the probe and plant. This condition is reached when no alternating current flows in the probe circuit. In the second method, air in the neighbourhood of a probe and plant surface is made conducting by α -particle irradiation and a current flows between probe and plant to bring them to electrical equilibrium.

The third method, which forms the subject of the present paper, will now be described in more detail.

A fortunate property of the root is its ability to grow strongly and healthily in aerated water. If the conductivity of the water is sufficiently low, potential differences between points in the water adjacent to the root can be observed, owing to the currents generated by the root e.m.f.'s. For this reason, it was decided to grow bean roots vertically in a 10^{-4} N KCl solution under controlled

conditions and observe the potential pattern in the water adjacent to the root and its relation to the rate of elongation of the root.

Since the measuring tubes are not in contact with the plant, no effects due to injury by them can occur. As the salt concentration in the tubes and in the bath is the same, the point of measurement on the plant is not in a different condition from neighbouring points resulting from local diffusion or other local variations.

Errors introduced in the measuring circuit have been prevented by the use of a suitable valve electrometer and mercury-calomel electrodes. The whole input circuit is insulated with polystyrene and shielded to prevent A.C. pick-up. The plants are left in the measuring bath for at least an hour before measurements are begun to avoid stimulation effects which may occur in setting up the plant for measurement. The bath is aerated, stirred, and its temperature controlled to avoid changes in environment. No effect was observed on either rate of growth or the potential pattern of bean roots due to light.

As the potential measured is an ohmic drop along a current, its source is evidently an active e.m.f. involving energy change in the plant organ.

IV. MATERIAL

For experiments described in this paper, the broad bean, *Vicia faba* L., Johnson's Long Pod variety, was used. After soaking the seeds overnight in water, the seed coats were removed and a number of seeds impaled on stainless steel rods 1/16 in. dia. Mounted in this way, the seedlings could be handled easily for growing in water-baths and could be transferred to the measuring bath with a minimum of stimulation. Impaling the seed did not appear to affect the development of the plant in any way.

For most experiments the beans were prepared by growing in baths of aerated distilled water at 25°C. The water in the tank was slowly changed from a reservoir tank. The rods supported the plants so that the shoot was above the water-level and the roots were submerged and grew vertically. Plants 2-3 days old with roots 20-30 mm long were used in the experiments.

In one series of experiments discussed later, the roots were prepared by growing in air saturated with water vapour and minute water droplets. The fine spray was produced by a simple atomizer using compressed air. In this bath the roots grew quite strongly but the root surface appeared rather different from that of a root grown in water and more like what is observed for a root grown in dry soil or sphagnum moss.

V. APPARATUS

The "Perspex" measuring bath is shown in Plate 1. It was 7 in. long, 6 in. wide, and 3 in. deep and was insulated with polystyrene. The bath was aerated and the water in the tank circulated by passing compressed air through a sintered glass plug *P* mounted in the tank.

Heating the bath presented some problems. Electrical interference had to be avoided and an arrangement giving rapid response to the thermostat must

be used. It was decided to heat the bath by means of a coil of nichrome wire stretched across the top of the bath just above water-level. A 12-V A.C. supply was connected to the coil and heat radiated downwards warmed the water. Surprisingly little A.C. pick-up was experienced, and this arrangement, with some shielding of the heater from the measuring circuit, proved very satisfactory. The thermostat was a mercury-alcohol switch, C, which controlled the heater through a relay. Temperature stability was $\pm 0.5^{\circ}\text{C}$.

The 10^{-4}N KCl solution used in the measuring bath was changed from a reservoir. The water was made to drip into the bath, and to drip out at the overflow to facilitate electrical insulation of the bath.

The beans were set up in the measuring tank on a stainless steel rod passing through the cotyledon with only the roots immersed and held vertically.

The electrometer used in these experiments employed a pair of matched M.E. 1400 Mullard electrometer valves in a balanced circuit. The instrument was designed to be insensitive to fluctuations in the high tension voltage and in the valve heater current. A Cambridge spot galvanometer (full scale deflection about $2\ \mu\text{A}$) was used and the maximum sensitivity of the electrometer was such that an input of 1 mV produced a deflection of 3 cm on the scale. Currents flowing in the input circuit of the electrometer under the conditions of operation were not greater than $10^{-12}\ \text{A}$.

The connections to the measuring bath were made using mercury-calomel electrodes. The reference electrode was connected directly to the bath. The other electrode made contact with the bath through a measuring tube, T, containing 10^{-4}N KCl agar. This tube was mounted on a micromanipulator, M, allowing it to be moved easily in the vicinity of the plant root. Since 10^{-4}N KCl has a low conductivity (about $1.5 \times 10^{-5}\ \text{mho cm}^{-1}$ at 25°C) the tip of the measuring tube had to be coarse or hand capacity effects became troublesome owing to the high resistance of the input circuit. In most of these experiments the diameter of the tip was about 0.5 mm, corresponding to a tip resistance of about 15 M Ω .

Elongation of the root was measured by projecting a greatly enlarged image on a wall. A 5-in. Waterworth projection lens was used, mounted in front of the box with its axis horizontal. For most experiments back lighting was employed, giving an enlarged shadow of the root, but in some experiments the root was marked, and strongly illuminated from the front so that the regions of elongation of the root could be found. In this way, changes in length of the order of 0.02 mm could be observed.

The whole box could be moved laterally on a metal slide, G, so that each bean in the box could be placed in turn in front of the lens for measurement.

VI. RESULTS

The present paper consists of a study of the potential pattern close to a bean root actively growing in water contrasted with that which appears when growth is inhibited.

The aspect of growth that has been selected is rate of elongation and attempts have been made to control this by a variety of means, and study the resulting potential changes.

Several treatments have been applied to the growing root, such as subjecting it to mechanical vibration, controlling its oxygen supply, growing it at varying temperatures, and inhibiting growth by the application of a suitable concentration of auxin.

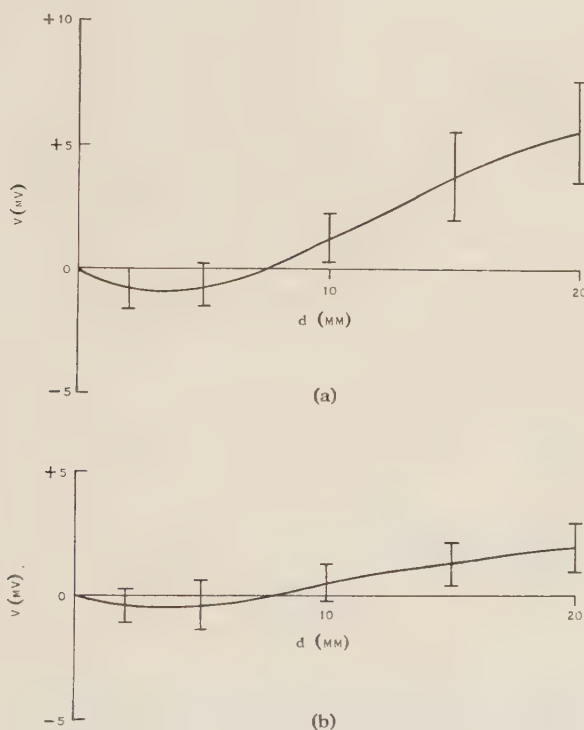


Fig. 2.—Control of growth and potential pattern of bean roots by the application of indoleacetic acid (IAA). (a) Roots at 25°C untreated with IAA. Mean growth rate 0.82 ± 0.060 mm/hr. (b) Same roots at 25°C 2 hr after treating with IAA (2 mg/l). Mean growth rate 0.027 ± 0.077 mm/hr. V is the potential relative to that of the root tip and d is the distance along the root from the tip.

The last of these methods, auxin treatment, was considerably the most successful. By this means, elongation could be inhibited, and the inhibition subsequently removed and growth restored. The method of temperature control also provided results of interest which are described below, but preliminary results by the other methods were not so promising and study of these has not yet been followed up.

In both cases studied, the pattern of active e.m.f. was correlated with the rate of elongation. The fact that the correlation was the same in both cases suggests a direct link between elongation and potential pattern. Of course, it is possible that both elongation and potential pattern are more dependent upon some third change common to both methods of control.

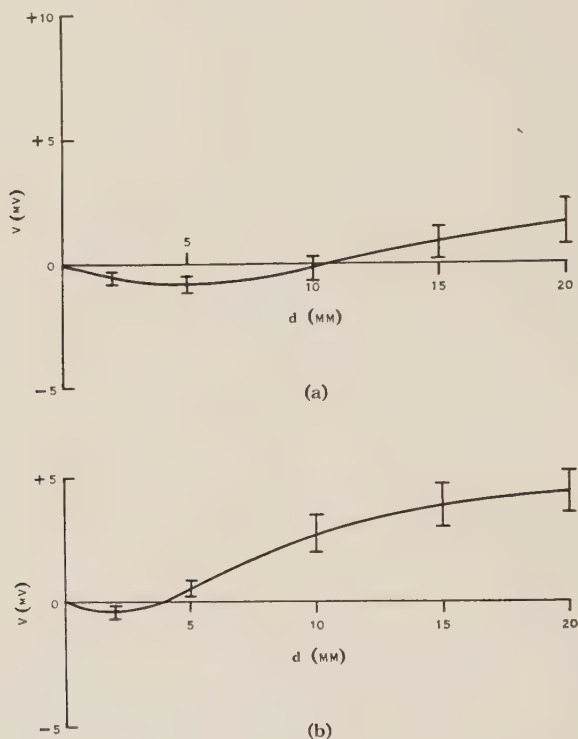


Fig. 3.—Recovery of growth and potential pattern on removal of IAA. (a) Roots at 25°C 2 hr after treating with IAA (2 mg/l). Mean growth rate 0.037 ± 0.043 mm/hr. (b) Same roots at 25°C 2 hr after removal of IAA from bath. Mean growth rate 0.36 ± 0.072 mm/hr.

(a) Control of Growth with Auxin

(i) Bean seedlings with roots approx. 25 mm long were transferred to the 10^{-4} N KCl measuring bath at 25°C and allowed to reach an equilibrium with their environment.

After about 2 hr, the potential pattern and rate of elongation of the roots were measured, and those whose growth rate was less than 0.4 mm/hr discarded. Indoleacetic acid (IAA) (2 mg/l of solution) was then added to the bath containing the rapidly-growing beans. After about an hour the potential pattern had reached a new steady state and the average potential and rate of elongation over the next 2 hr were recorded.

The results of this experiment are shown in Figure 2. In this and subsequent diagrams the vertical lines through the points mark the 95 per cent. confidence limits; that is, the probability is 0.95 that the mean of the population represented by the sample lies within the limits given by the ends of the line. The limits for the mean growth rate also are the 95 per cent. confidence limits.

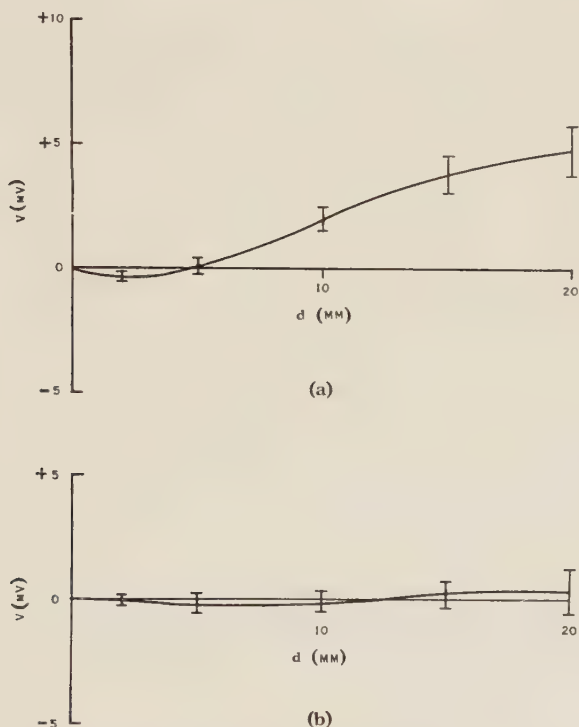


Fig. 4.—Control of growth and potential pattern by temperature. (a) Roots grown and measured in water at 25°C. Mean growth rate 0.532 ± 0.076 mm/hr. (b) Roots grown in saturated air and measured in water at 37°C. Mean growth rate 0.007 ± 0.041 mm/hr.

(ii) Bean seedlings were transferred to the measuring bath at 25°C containing 10^{-4} N KCl and 2 mg/l IAA. After about 2 hr elongation had practically ceased. The potential pattern was then observed, and the water in the bath replaced with KCl solution without IAA. At 1.3 hr after the removal of the IAA, when the root was again elongating, the mean potential pattern and growth rate were measured.

The results of this experiment are given in Figure 3.

An examination of Figures 2 and 3 shows that the inhibited roots have a markedly different potential pattern from those growing strongly. The normal potential pattern of a growing root shows a region 2.5 mm from the tip most negative, with the base of the root approx. 6 mV more positive. Suitable

treatment with IAA inhibits the elongation of the root and reduces the potential gradients along the root by a significant amount. Removal of the auxin allows the root to grow again (although the recovery is not complete in 2 hr, the growth rate being well below that of untreated roots) and the potential gradient along the root returns almost to its value prior to auxin treatment.

(b) Control of Growth by Temperature

Figure 4 (a) shows the normal potential pattern for roots grown in 10^{-4}N KCl at 25°C . In this case, and in the following one at 37°C , the growth and potentials were averaged for a 24-hr period following the initial settling down.

Figure 4 (b) shows the potential pattern for roots measured in 10^{-4}N KCl at 37°C . Inhibition of growth at this temperature was most marked in roots pre-treated by growth in a saturated atmosphere for 24 hr before exposure to 37°C . The graph refers to roots treated in this way. It is seen that the roots have almost entirely stopped elongating and the potential differences along the root are not significantly different from zero. Roots measured at this temperature for 24 hr and then returned to a 25°C bath do not recover normal growth. The activity of the primary meristem is suppressed and the main root stops growing, but pronounced initiation of secondary roots quite close to the primary root tip is observed (see Plate 2). Electromotive forces are once more produced by the root when it is returned to the 25°C bath, but they are not the same as those for a root which has not been treated at 37°C .

(c) Power and Current

More detailed experiments have been made from which the current density and current direction in the neighbourhood of the root can be deduced. In addition, the total power dissipated in the solution due to current produced by the root can be calculated.

These data have been obtained by measuring the radial and longitudinal components of the potential gradient in different orientations around the root. With this information, it is possible to map the equipotential surfaces and hence the current paths throughout the external medium. A typical simplified pattern is shown in Figure 5, in which the full lines are current paths and the dotted lines are equipotentials.

For a root growing actively in 10^{-4}N KCl at 25°C , the total current leaving the root (which, of course, must be equal to the total current entering it, since leakage paths have been eliminated) is of the order of 5×10^{-8} A. If the root is taken to have a surface area of 2.5 cm^2 immersed, the mean current through unit area is $4 \times 10^{-8} \text{ A/cm}^2$, although, of course, it varies considerably over the whole root surface. In certain regions of an active root it may be as high as $2 \times 10^{-7} \text{ A/cm}^2$. The total power dissipated in the surrounding medium is of the order of $2 \times 10^{-10} \text{ W}$.

It is found that small variations with time of the potential pattern occur even when all environmental factors known to affect the plant are controlled.

Under certain conditions, rapid rhythmic oscillations of potential take place. These oscillations can be induced experimentally.

Investigations of these phenomena are continuing and it is proposed to make them the subjects of later papers.

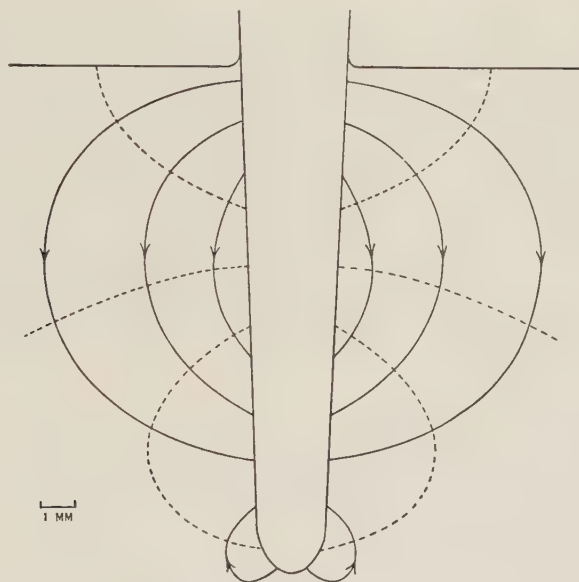


Fig. 5.—Typical simplified pattern of the current paths through the 10^{-4} N KCl solution in which bean root is growing. The full lines are typical current paths (lines of force) and the dotted lines are equipotentials.

VII. DISCUSSION

The experiments described in this paper were made with the object of providing definite reproducible information about the electric currents produced by a plant organ acting as a unit. The long-range object is to obtain information about the morphology and behaviour of the electric patterns that characterize integrated plant structures and their relationship to the morphology and development of the material structure.

It is found that the most negative part of the potential pattern for a rapidly growing root corresponds with the region which is elongating most rapidly (2-7 mm from the tip). A possible mechanistic explanation is now given.

The greatest uptake of salt might be expected to take place in the region of greatest rate of elongation, where newly vacuolated cells must fill up to a salt concentration which is many times greater than that of the external solution. It is known that the mobility of K^+ is greater than that of Cl^- in tissue (Hope 1951). The K^+ will penetrate the root more rapidly, forming an electric double layer, and a slight excess of Cl^- will build up in the region outside the elongating part of the plant. This will make the region electrically negative. With this

hypothesis it would also be expected that a root which had not elongated for some time would be absorbing salt at a slower rate and more uniformly over the whole root surface. Under these conditions the observed reduction in the external field is to be expected.

The mechanisms which produce electromotive forces in plant tissues may be such that some can supply more power than others. Whether the field produced by one of these will be observed outside the plant in a particular case depends on the magnitude of the current generated in the conditions of measurement. If this current is too large, the field may not be measured, either because the e.m.f. itself is polarized, or because the potential drop in the tissue is so great that the external field is insignificant.

The present method selects for measurement only those bioelectric processes which are capable of supplying a relatively large amount of power to the external medium. By employing other methods which were mentioned earlier in this paper (Section III) it is possible to investigate less powerful bioelectric processes taking place in the organ and so gain a more complete picture of the bioelectric behaviour of plants.

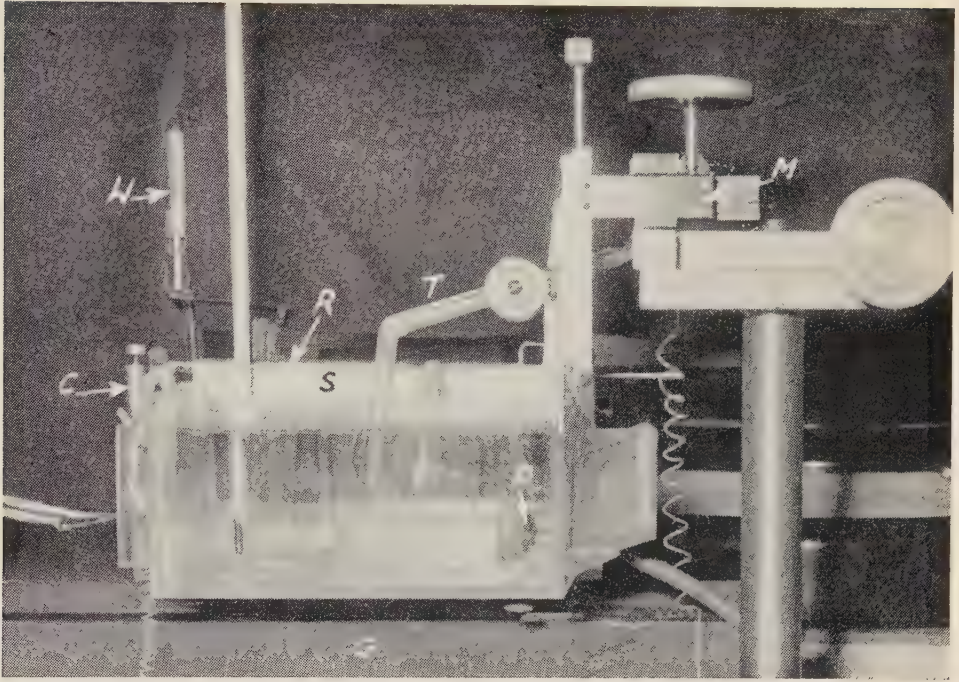
VIII. ACKNOWLEDGMENT

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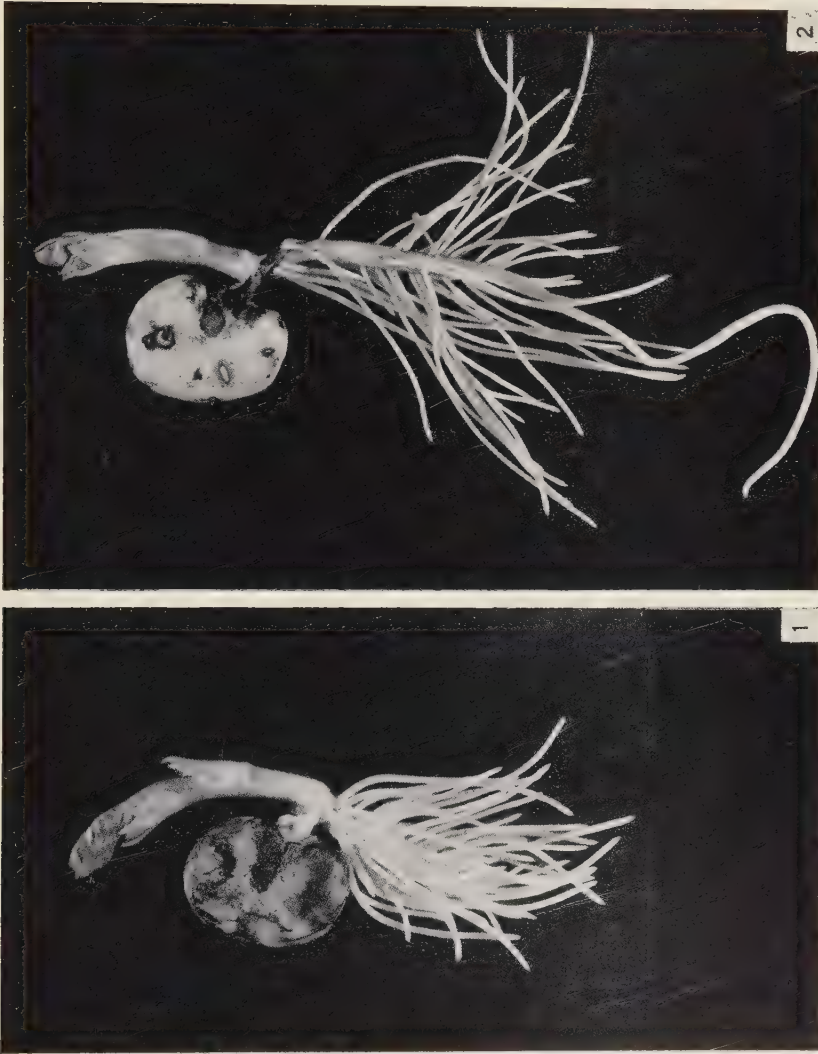
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ELECTRIC CURRENT GENERATED BY ROOT GROWTH



The measuring bath used in the experiments described in the paper. The following parts are referred to in the text: *P*, sintered glass plug; *S*, shield for A.C. heater; *C*, mercury-alcohol switch control; *R*, stainless steel rod supporting plant; *M*, micromanipulator; *T*, measuring tube; *W*, water inlet; *G*, metal slide for moving box.

ELECTRIC CURRENT GENERATED BY ROOT GROWTH



Abnormal subsequent growth of roots treated for 24 hr at 37°C.
Fig. 1.—Root one week after treatment at 37°C. Notice lack of development of primary root with secondary roots appearing quite close to primary tip.
Fig. 2.—Untreated root of same age.

THE EFFECT OF NITROGEN ON ZINC DEFICIENCY IN SUBTERRANEAN CLOVER

By P. G. OZANNE*

[Manuscript received September 9, 1954]

Summary

Data are presented from four sand culture and one water culture trial.

Subterranean clover plants growing on a soil low in zinc showed increased severity of zinc deficiency symptoms when the nitrogen supply was increased. This effect was produced by applications of NaNO_3 , L-asparagine, NH_4NO_3 , and $(\text{NH}_4)_2\text{SO}_4$. It was not due to changes in soil pH, nor to increased growth demand arising from applied nitrogen.

Under conditions of low zinc supply, the zinc concentration in roots was found to be correlated with the percentage protein N present. The proportion of total zinc absorbed that was translocated to the plant tops was also found to be related to the percentage protein N in the roots.

It seems likely that increased nitrogen supply caused more of the zinc to be retained by the roots in zinc protein complexes. Under conditions of low zinc supply, this root retention led to severe symptoms of zinc deficiency in the plant tops.

I. INTRODUCTION

Responses to zinc-containing fertilizers are obtained over wide areas in the south-west of Western Australia, and have been reported by Teakle (1942), Dunne and Throssell (1948), Rossiter (1951a), and others. A feature of this zinc deficiency is that, in the same locality, the symptoms vary in severity from season to season and from year to year. During a study of the influence of light intensity on this varying response (Ozanne, unpublished data), it became apparent that the zinc requirement of subterranean clover is markedly affected by the supply of nitrogen available to the plant. As subterranean clover is an important component of some pastures in Western Australia, this zinc-nitrogen relationship was examined further.

Haas (1936), Chapman, Vanselow, and Liebig (1937), and Camp and Fudge (1945) mentioned that liberal nitrogen applications may produce zinc deficiency symptoms on citrus plants growing with a low zinc supply. These authors suggested that this zinc deficiency was partly induced by the increased growth with high nitrogen, and also partly by the rise in pH of the culture medium when NaNO_3 was used as a nitrogen source.

Reuther and Smith (1950), also working with citrus, reported a trial in which increasing levels of applied nitrogen led to increasingly severe symptoms of zinc deficiency. They suggested a nitrogen-zinc ratio effect on symptom

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expression; but, as fruit yields increased considerably with each increase in added nitrogen, the crop demand must also be taken into account.

In this paper, nitrogen supply and nitrogen content are examined with regard to their effects on the growth and zinc content of subterranean clover.

II. EXPERIMENTAL METHODS

(a) Soil Culture

Subterranean clover (*Trifolium subterraneum* L.) (Dwalganup strain) was grown from commercial seed inoculated with an effective strain of *Rhizobium*. The plants were grown in white glazed porcelain pots containing 2 kg of Muchea Sand which field trials by Rossiter (1951a) have shown to be deficient in zinc. This grey siliceous sand is too acid (pH 5.2) for good nodulation by subterranean clover and lime was mixed through the soil to raise the pH to about 6.7.

The following basal dressing (mg per pot) was applied at sowing: K_2SO_4 , 210; $Ca(H_2PO_4)_2 \cdot H_2O$, 51; $MgSO_4 \cdot 7H_2O$, 70; $MnSO_4 \cdot 4H_2O$, 15; $FeSO_4 \cdot 5H_2O$, 15; $CuSO_4 \cdot 5H_2O$, 15; $(NH_4)_6 Mo_7 O_{24} \cdot 4H_2O$, 1.5; H_3BO_3 , 6. To keep some iron in solution, 15 mg of tartaric acid were added. In Experiments 1-4, 140 mg/pot is equivalent to 1 cwt/ac on an area basis.

All nutrients used were prepared from "analytical reagent" grade chemicals. "Pyrex" glass-distilled water only was used for watering the plants.

In each trial a factorial experimental design was used. To half the pots $ZnSO_4 \cdot 7H_2O$ was added at 35 mg per pot to give a set of treatments with ample zinc.

In the absence of applied zinc, the plants showed zinc deficiency symptoms similar to those described by Rossiter (1951b) and Millikan (1953). In Experiments 1 and 3 the severity of the symptoms was recorded for each pot by counting the leaves present and expressing the number showing definite symptoms as a percentage of the total.

The tops and roots of all plants were harvested separately about 42 days after germination and while the plants were still in the vegetative stage of growth. At harvest the plant roots were washed from the soil, rinsed in diluted acetic acid, and then rinsed thoroughly in distilled water before being oven-dried at 85°C. The data presented in Tables 1-3 are mean values obtained from six pots.

Experiment 1.—Commencing at germination, 280 mg per pot of $NaNO_3$ were added at fortnightly intervals to the "high-nitrogen" treatments.

Experiment 2.—L-Asparagine monohydrate at 70 mg per pot was applied weekly to the high-nitrogen treatments as a source of nitrogen.

Experiment 3.— NH_4NO_3 (280 mg per pot) was applied at sowing to the high-nitrogen pots.

Experiment 4.—Four nitrogen treatments were used: no applied nitrogen; $NaNO_3$ at 70 mg per pot; $(NH_4)_2SO_4$ at 55 mg per pot; and L-asparagine at 62 mg per pot.

The latter three treatments were applied weekly and contained equivalent amounts of nitrogen.

(b) Water Culture

In Experiment 5, the subterranean clover plants were grown in 3-l. "Pyrex" beakers filled with a basal solution containing the following ions (mM per beaker): K^+ , 8.5; Ca^{++} , 2.5; Mg^{++} , 2.0; NO_3^- , 5.0; $H_2PO_4^-$, 3.0; $SO_4^{=}$, 4.0; also the following elements (p.p.m.) Cu, 0.02; Mn, 0.50; B, 0.50; Mo, 0.01; Fe, 0.50 p.p.m. in sequestered form with "Versene."

Three levels of N supply were obtained by adding NH_4NO_3 at nil, 5mM and 15mM per beaker. The two levels of zinc added were nil. and 30 μg per beaker.

Stock solutions of the major elements were saturated with H_2S to remove zinc impurities. All culture solutions were kept at approximately pH 6.4.

TABLE 1

EFFECT OF NITROGEN AND ZINC SUPPLY ON YIELD AND COMPOSITION OF PLANT TOPS, EXPERIMENT 1.
VALUES GIVEN REPRESENT MEANS FROM 6 POTS

Treatments	Dry Weight (g)	Increase in Weight with Zn (g)	No. of Leaves with Symptoms (%)	Protein N in Tops (% dry wt.)	Zn in Tops (p.p.m.)
Low Zn, low N	1.91	—	34	2.05	25.0
Low Zn, high N	1.08	—	84	2.91	17.0
High Zn, low N	2.70	0.79	—	2.20	71.3
High Zn, high N	2.97	1.89	—	2.22	64.6
L.S.D. of two means at $P = 0.01$	0.77	1.03	—	—	—

(c) Chemical Analysis

Zinc determinations were made using the wet digestion method of Piper (1944) and a modification of the photometric technique described by Cowling and Miller (1941).

Protein N was determined by the micro-Kjeldahl method. In Experiments 1, 3, and 4, the fresh plant material was dried at 85°C, and then the protein N precipitated from a trichloroacetic acid solution at pH 4.5. In Experiment 5, the freshly harvested plant roots were rinsed, then immersed in boiling 80 per cent. alcohol for 1 min. After standing overnight in the 80 per cent. alcohol, the insoluble material was filtered off and protein N determined in it. Soluble zinc was determined in the filtrate.

III. RESULTS AND DISCUSSION

(a) Effect of Nitrogen Supply on Growth and Zinc Content

Experiment 1.—Previous experience has shown that clover readily uses $NaNO_3$ as a source of nitrogen, and in Experiment 1 the increase in protein

N shows that the nitrate was assimilated (Table 1). Early in this trial the plants nodulated well and, where zinc was added, the growth response to nitrogen was small.

In the absence of applied zinc, plant growth was markedly reduced by the addition of nitrogen. Decreased growth was associated with increased severity of zinc deficiency symptoms, and reduced zinc concentration in the plant tops.

In this experiment the addition of NaNO_3 caused a rise in soil pH from 6.65 to 7.15.

Experiment 2.—The treatments here were similar to those in Experiment 1 but an attempt was made to prevent the possible effects of change in soil pH on zinc absorption by using L-asparagine as a source of nitrogen. Ghosh and Burris (1950) and Vantsis and Bond (1951) reported that clover is able to use nitrogen from L-asparagine. This compound was applied to the "high

TABLE 2

INTERACTION OF NITROGEN WITH ZINC RESPONSE, EXPERIMENT 2. VALUES GIVEN REPRESENT MEANS FROM 6 POTS

Treatments	Dry Weight (g)	Increase in Weight with Zn (g)	Zn in Leaves (p.p.m.)
Low Zn, low N	1.06	—	34.1
Low Zn, high N	1.15	—	22.7
High Zn, low N	2.24	1.18	120.1
High Zn, high N	5.13	3.98	132.6
L.S.D. of two means at $P = 0.01$	0.22	0.31	—

nitrogen" pots and gave a large growth response in the presence of zinc (Table 2). No differences in soil pH were caused by the addition of asparagine. At the low zinc level, on the other hand, additional nitrogen gave an insignificant change in plant size. However, the amount of zinc present in the plant leaves was greatly reduced.

Experiment 3.— NH_4NO_3 , used as nitrogen source, gave the same marked nitrogen by zinc interaction as did asparagine in Experiment 2 (Table 3). The soil pH was unchanged by the added NH_4NO_3 .

In this trial the plant roots were analysed for zinc as well as the tops. Surprisingly, the "low Zn, high N" plants, showing severe symptoms, were found to contain more zinc in the roots than the "low Zn, low N" plants. Even under ample zinc conditions, where added nitrogen gave considerably larger plants, it also caused greater root concentration of zinc.

In Experiments 1, 2, and 3, when the nitrogen status of low zinc plants was increased, more severe symptoms developed and zinc content of the aerial parts was reduced. High nitrogen supply must then have (1) reduced zinc absorption by the roots, or (2) reduced translocation of absorbed zinc to the

plant tops. The increased zinc content of "high N" roots in Experiment 3 indicates that the latter cause was the most likely.

(b) *Zinc Distribution in Relation to Protein Nitrogen*

Jacobson and Overstreet (1947) suggested that absorbed ions are fixed in the plant cells in the form of chemical compounds which are stable in living tissue but quite labile in dead. They mentioned proteins, amino acids, and organic acids as forming such chelated compounds, especially with polyvalent cations.

TABLE 3

EFFECT OF NITROGEN ON YIELD, ZINC CONTENT, AND ZINC DEFICIENCY SYMPTOMS, EXPERIMENT 3.
VALUES REPRESENT MEANS FROM 9 POTS

Treatments	Total Tops Dry Weight (g)	Increased Weight with Zn (g)	No. of Leaves with Symptoms (%)	Protein N* (% dry wt.)	Zn Content	
					Leaves (p.p.m.)	Roots (p.p.m.)
Low Zn, low N	0.550	—	33	1.69	22.9	132
Low Zn, high N	0.485	—	63	2.20	21.5	162
High Zn, low N	0.928	0.378	—	1.51	60.3	210
High Zn, high N	1.350	0.865	—	1.72	70.6	359
L.S.D. of two means at $P = 0.01$	0.225	0.318	—	0.15	2.9† 11.1‡	25† 36‡

* Protein nitrogen in stems and petioles.

† Low Zn.

‡ High Zn.

In Experiment 3, increased nitrogen supply gave rise to increased protein N, and also increased retention of zinc within the roots. It was considered likely that protein compounds in the roots may have formed poorly dissociated chelate complexes with some of the absorbed zinc, and thus prevented its translocation to the plant tops. Two trials were carried out to investigate this possibility.

Experiment 4.—Here plants using the various nitrogen sources NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$, and asparagine were compared with plants relying solely on atmospheric N made available after symbiotic fixation in the root nodules. As in the previous trials, increased nitrogen supply from all sources gave increased zinc response.

After harvest the plant material was dried and the protein compounds precipitated from solution by trichloroacetic acid. Zinc determinations were made on both the precipitated material and on the soluble filtrate. Only some 12 per cent. of the total zinc in the plant roots was found to be precipitated with the protein material using this method.

To see if a relationship existed between the protein content and zinc content of the plant roots, the percentage protein N found was graphed against the p.p.m. total zinc present. As may be seen from Figure 1, the concentration of zinc present in the plant roots was positively correlated with the percentage protein N.

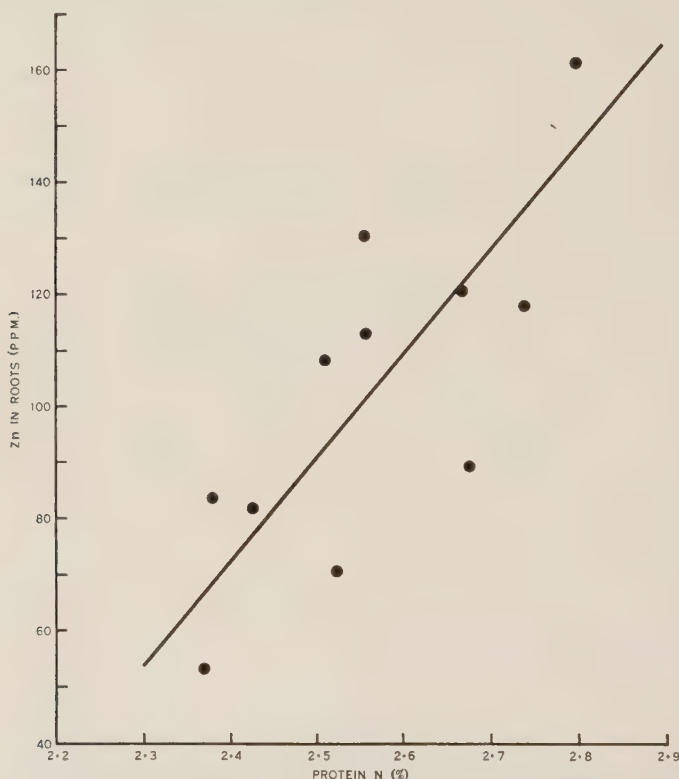


Fig. 1.—Relationship of protein nitrogen to concentration of zinc present in plant roots. The line drawn from the regression equation $y = -370 + 184x$; and $r = 0.81$ significant at $P = 0.01$.

Only values from "low Zn" roots were included for it was thought that the relationship might be different under the conditions of luxury absorption with "high Zn." Although all plants had the same low zinc supply, those roots which formed more protein also contained more zinc. The work of Bean (1940), Wood and Sibly (1952), and others has shown that zinc is necessary for protein synthesis. However, Glasstone (1947) found a very low zinc requirement for tomato roots. Also experiments by the author have shown that clover roots may continue to grow in an almost zinc-free medium without decrease in protein N. These observations suggest that the requirement of zinc by subterranean clover roots is at least no higher than the requirement of the tops, and that the relatively high concentrations of zinc found in the roots serve

no useful purpose. It was shown in Figure 1 that increased protein N is associated with increased zinc in the roots. Figure 2 shows that protein N is closely related to the power roots have to retain zinc at the expense of the plant tops.

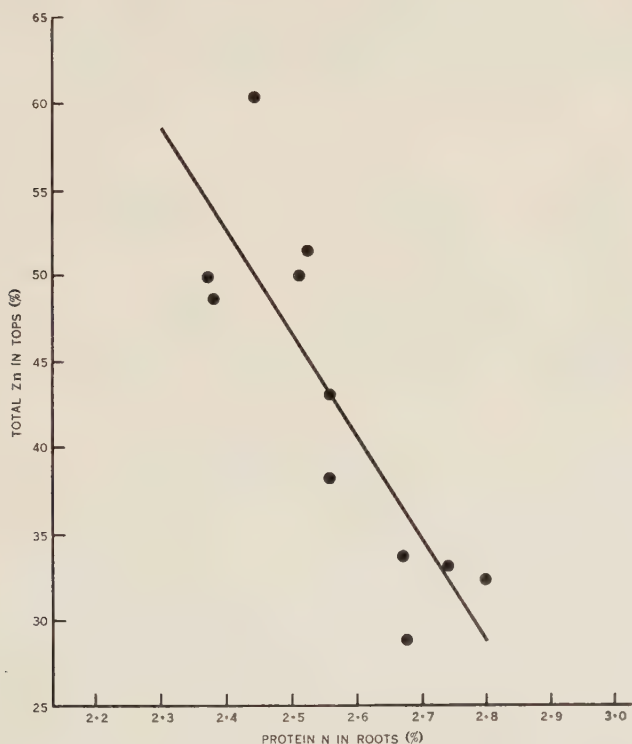


Fig. 2.—Relationship between concentration of protein nitrogen in the roots and the proportion of the total absorbed zinc translocated to the plant tops. The line is drawn from the regression equation $y = 196 - 59.7x$; and $r = -0.85$ significant at $P = 0.001$.

As so little of the zinc in the plant roots was precipitated from solution with the protein material the possibility remained that the zinc had not formed complexes with the root proteins, but perhaps with some other compounds which varied in concentration with protein N.

Experiment 5.—In this trial the plants were grown in culture solutions containing three levels of nitrogen supply and two of zinc. At harvest, fresh weights were taken and aliquots of the plant roots were killed immediately in boiling 80 per cent. ethanol. After standing, protein N and insoluble zinc were determined on the precipitate from this solution and soluble zinc from the filtrate plus washings.

Using this less severe technique, 75 per cent. of the root zinc was recovered from the protein precipitate, compared with the 12 per cent. in Experi-

ment 4. This suggested that the root zinc is held in a labile metallo-organic complex which was largely decomposed by the analytical technique used in Experiment 4. The small amounts of soluble zinc obtained from the roots of Experiment 5 were expressed as p.p.m. of the oven-dry root weights and graphed against percentage protein N as shown in Figure 3. The close positive correlation between soluble zinc and protein N suggests that this fraction of the zinc was also linked to the protein compounds of the living plant.

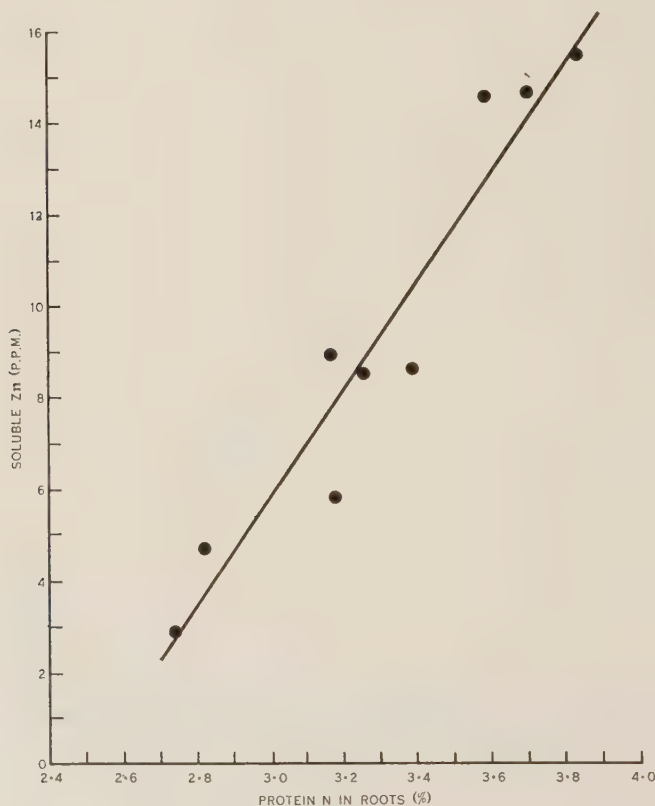


Fig. 3.—Relationship of soluble zinc to protein nitrogen precipitated from solution by boiling 80 per cent. ethanol. The line is drawn from the regression equation $y = -29.3 + 11.7x$; and $r = 0.96$ significant at $P = 0.001$.

Thus it seems probable that all the zinc present in the plant roots was in combination with the root protein in the living plant. As roots can retain relatively high concentrations of zinc while the tops are suffering severely from zinc deficiency it might be expected that very little soluble or uncomplexed zinc would be present in roots when the zinc supply is low.

From the experiments described here it was concluded that increasing the nitrogen supply available to subterranean clover causes more of the absorbed zinc to be retained by the roots in a poorly dissociated zinc-protein complex.

Under conditions of low zinc supply, this increased root retention is able to cause severe symptoms of zinc deficiency in the plant tops.

It is of interest to compare the results described above with recent work on copper accumulation by plants. Seymour (1951) found that tung plants given a high nitrogen supply did not translocate copper to the leaves. From work on citrus trees, Smith (1953) reported that copper accumulation by citrus roots appears to be directly related to the non-replaceable protein nitrogen of the roots.

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THE EFFECT OF SEED EXTRACTS ON THE INFECTIVITY OF PLANT VIRUSES AND ITS BEARING ON SEED TRANSMISSION

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Summary

Substances inhibiting infection by cucumber mosaic and tobacco mosaic viruses have been found to occur in the seeds of some of their hosts.

The following evidence is considered to show conclusively that the action of these substances is to inhibit infection rather than to inactivate the viruses:

- (1) The substances are capable of instantaneous effect.
- (2) Their effect is dependent upon the host used.
- (3) Non-infective mixtures of inhibitor and inoculum can be made infectious by dilution.
- (4) Their effect is greatest on concentrated inocula.
- (5) They will affect lesion production when applied up to 2 days before inoculation.
- (6) The inhibitor from cucumber seeds affects lesion production when applied to the under surface of cowpea leaves.

Both of the inhibitors are proteins.

The bearing of these results on the theories that have been advanced to explain the rarity of seed transmission of plant virus diseases is discussed.

I. INTRODUCTION

The rarity of seed transmission of plant virus diseases, and particularly of some which are highly infectious, has long lacked an adequate explanation. Bennett (1936) advanced a very satisfactory explanation for the lack of seed transmission of beet curly top virus. He showed this virus to be chiefly restricted to the vascular tissues of its hosts and thus unable to invade the embryos of the seeds. This explains satisfactorily the lack of seed transmission of all plant viruses that are restricted to the vascular tissues of their hosts, but it does not account for the rarity of seed transmission of a far greater number of plant virus diseases which are unrestricted in the tissues of their hosts and which can move quite independently of vascular tissue. Bennett, assuming that plant viruses spread through plant tissues only via plasmodesmata, suggested that these viruses are not seed-transmitted because of the lack of plasmodesmatal connections from the embryo to the parent plant. This suggestion also assumes that those viruses which are seed-transmitted are capable of between-cell movement other than through plasmodesmata.

Recently, Caldwell (1952), working with aspermy disease of tomato, in which infected plants produce few seeds and less than 1 per cent. of normal pollen, suggested: "The presence of virus in the microspore-mother-cell results

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in a complete interference with the normal stages of meiosis . . . ” This hypothesis could well explain the lack of seed transmission of plant viruses which cause a reduction in the amount of seed and pollen produced, but there are many plant viruses which induce no such effect.

Attempting to explain the lack of seed transmission of tobacco mosaic virus Duggar (1930) suggested that the virus was inactivated by some “specific protein or other specific material” in the seeds. Experimental evidence in support of this theory was published by Kausche (1940) who showed that the addition of aqueous extracts of tobacco seed to purified tobacco mosaic inoculum could reduce its infectivity by as much as 50 per cent. Kausche suggested that the inactivator has a surface effect on the tobacco mosaic virus molecule which makes it non-infective, but his results can be equally well interpreted as demonstrating the existence in tobacco seed of a substance or substances capable of inhibiting infection of *Datura stramonium* by tobacco mosaic virus.

Work described here has been carried out to determine whether inhibitors or inactivators of plant viruses do exist in the seeds of their hosts, and to determine the nature of their action.

II. MATERIALS AND METHODS

The strain of cucumber mosaic virus used was obtained from Mr. L. L. Stubbs, of the Department of Agriculture, Victoria. Inoculum for all experimental work with cucumber mosaic virus was obtained from 3-week old cucumber seedlings within 10 days of their being inoculated. The seedlings were ground in composite phosphate buffer at pH 7, filtered through muslin, and diluted with more buffer until the preparation consisted of 1 volume of infective sap in 24 volumes of buffer. All cowpea seedlings used for assay of cucumber mosaic virus were of the variety Blackeye from seed of Californian origin. The technique used for inoculating cowpeas has been described previously (Crowley 1954). All experiments carried out with cowpeas involved either two or four treatments. Those with two treatments were carried out by whole leaf comparisons, using 20 replicates. The experimental points in Figures 1 and 3 were obtained in this manner. Experiments involving four treatments were carried out by the half-leaf technique with 16 replicates of each treatment in a randomized block design arranged so that each treatment occurred on every plant, and occurred an equal number of times in each leaf position. Extracts of seed tissues used in work with cucumber mosaic virus, except where otherwise stated, were prepared by grinding 1 g of tissue in 20 ml of distilled water and filtering through muslin before use. Five ml of this extract were then added to 5 ml of virus inoculum.

The inoculum used in all work with tobacco mosaic virus was obtained by diluting a sample of purified virus preparation (kindly supplied by Dr. R. J. Best) in 1000 volumes of composite phosphate buffer at pH 7. All *Nicotiana glutinosa* plants used were raised in a warmed insect-proof glass-house, and when approx. 10 weeks old were dusted with carborundum powder and inoculated with a glass spatula. All experiments with tobacco mosaic virus were

carried out by the half-leaf technique, all treatments occurring an equal number of times on left and right halves. Twenty or more replicates were used in all experiments. The experimental points in Figures 2 and 4 are means of 24 replicates, six, each with four leaves, being used for each dilution. The eight treatments used to obtain the results presented in Table 7 were each replicated 40 times in five 8×8 latin squares in which each treatment occurred on every plant and occurred an equal number of times in each leaf position. Extracts of tobacco seed used in work with tobacco mosaic virus were prepared

TABLE 1
EFFECT OF SEVERAL DILUTIONS OF AQUEOUS CUCUMBER EMBRYO EXTRACTS ON LESION
PRODUCTION BY CUCUMBER MOSAIC VIRUS ON COWPEA

Extract From	Mean Number of Lesions per Half Leaf			
	Material Added to Inoculum			
	Extract	Extract/10	Extract/100	Control Dist. Water
Cucumber embryos	0***	8***	71***	176
Cucumber testas	0***	6***	90**	138
Wild cucumber embryos	2***	136*	171	182
Wild cucumber testas	55***	152	147	152

* Difference from control significant at $P = 0.05$.

** Difference from control significant at $P = 0.01$.

*** Difference from control significant at $P = 0.001$.

by grinding 2 g of Blue Prior tobacco seed in 10 ml of phosphate buffer at pH 7 over a period of 3-5 hr and then filtering through muslin. After it was found that the inhibitor was heat-stable the extract was partially purified by heating in boiling water for 10 min and again filtering. Portions of this extract were then added to equal volumes of virus inoculum.

III. EXPERIMENTAL

(a) Effect of Seed Extracts on Plant Viruses

Two viruses, tobacco mosaic and cucumber mosaic, were selected for this work; tobacco mosaic virus because it had been used for similar work by Kausche, and because of all the plant viruses known the rarity of seed transmission of the virus is possibly the most puzzling; cucumber mosaic virus because it provided an example of a highly infectious plant virus, seed-transmitted in one host (wild cucumber, *Echinocystus lobata*) and not seed-transmitted in another (cucumber, *Cucumis sativa*). It was hoped that a comparison of

the results of work using seed extracts of these two hosts on one virus where seed transmission occurred in only one of the hosts would give an indication of the validity of Duggar's theory of inactivation by seed extracts. However, recent trials at the Waite Institute have shown that the seed transmission of cucumber mosaic virus in wild cucumber is much less frequent than reported by Doolittle and Gilbert (1919). Only one infected seedling occurred amongst more than 500 seedlings raised from seeds of infected plants.

The results of experiments carried out with both of these viruses are shown in Tables 1 and 2.

TABLE 2
EFFECT OF BUFFERED AQUEOUS EXTRACT OF TOBACCO
SEED ON LESION PRODUCTION BY TOBACCO MOSAIC
VIRUS ON *N. GLUTINOSA*

Treatment	Number of Lesions per Half Leaf
Inoculum + buffer	141
Inoculum + extract	37***

*** Difference significant at $P = 0.001$.

The results demonstrate the existence of some water-soluble constituent in seeds which can greatly reduce the number of lesions produced by both cucumber mosaic and tobacco mosaic viruses on their respective hosts.

With cucumber mosaic virus, embryo extracts were much more effective than testa extracts in reducing the number of lesions produced on cowpea. Cucumber extracts were regularly found to be more effective than wild cucumber extracts.

With tobacco mosaic virus, whole seeds were used as the small size of seeds did not permit the dissection of large numbers. The addition of tobacco seed extract to tobacco mosaic virus inoculum consistently resulted in a significant reduction in the number of lesions produced; but the extracts used here were four times as concentrated as those reported by Kausche to produce a similar effect. This is attributed to the fact that Kausche used a different variety of tobacco seed.

(b) Nature of the Action

It is impossible to tell in any simple manner whether a treatment which reduces the number of lesions produced by a given virus inoculum produces its effect by an effect on the host used for measuring infectivity, or by inactivating the virus. A number of ways in which the two processes can be distinguished have been suggested by several workers (Caldwell 1935; Slagle, Wolcyz, and Price 1952; Bawden and Freeman 1952). Inhibitors, whose effect is primarily to affect the susceptibility of the host used, can be distinguished from virus inactivators by possessing the following characteristics: they are capable of instantaneous effect; their effect is dependent on the host used;

they have greater effect on concentrated inocula; the effect is diminished by dilution; they have an effect when applied prior to inoculation or when applied to the under surface of leaves.

TABLE 3

INSTANTANEOUS EFFECT OF CUCUMBER EMBRYO EXTRACT IN INHIBITING LESION FORMATION BY CUCUMBER MOSAIC VIRUS ON COWPEA

Inoculation Time	Mean Number of Lesions per Half Leaf	
	Extract	Control
Immediately after mixing	9.8	94.4
4 Hr after mixing	4.8	24.6

The experiments described below, using all these methods, were carried out to determine the nature of the action of aqueous extracts both from cucumber embryos and tobacco seed.

TABLE 4

INSTANTANEOUS EFFECT OF TOBACCO SEED EXTRACT IN INHIBITING LESION FORMATION BY TOBACCO MOSAIC VIRUS ON *N. GLUTINOSA*

Inoculation Time	Mean Number of Lesions per Half Leaf	
	Extract	Control
Immediately after mixing	26.9	85.1
5 Hr after mixing	22.2	84.7
Immediately after mixing	26.4	94.9
24 Hr after mixing	20.1	89.1

(i) *Reaction Time*.—The results in Tables 3 and 4 show that the effect of both of the extracts used is just as great immediately (within 30 sec) after addition to the inoculum as it is several hours later. The decline in the infectivity of the treatments was consistently found to be of the same order as the decline in the infectivity of the controls, and was not more than normally occurs through a decline in the infectivity of inoculum with age.

(ii) *Effect of Host Used*.—The results of a typical experiment in which four different hosts were used to measure the infectivity of the inocula are set out in Table 5 and show that the effect of cucumber embryo extract is dependent on the host used for infectivity measurements. It is not thought possible

that these differences in the results are due to differences in the susceptibility of the different hosts, as in trials carried out concurrently with this work the dilution end-point of inoculum was found to be 1 in 10,000, using both cucumbers and cowpeas.

Similar trials could not be carried out with tobacco mosaic virus because no local lesion host other than *N. glutinosa* was available and even on this host it was impossible to inhibit local lesion production completely. Hence the use of any host producing systemic symptoms on infection would be futile, because 100 per cent. infection would always result.

TABLE 5

INFECTIVITY OF MIXTURES OF CUCUMBER MOSAIC VIRUS AND CUCUMBER EMBRYO EXTRACTS TO DIFFERENT HOSTS

	Host			
	Cowpea	Cucumber	<i>N. glutinosa</i>	Tobacco
	Mean Number of Lesions per Leaf	Proportion of Plants Infected		
Treatment	0	20/20	2/2	1/2
Control	69	20/20	2/2	2/2

(iii) *Dilution of Extract-Inoculum Mixtures*.—Gupta and Price (1950), in studying the nature of the effect of fungal extracts on plant viruses, showed that non-infective mixtures could be made infective simply by dilution and they concluded that the fungal extracts did not inactivate the virus but that “either the inhibitory agent enters into a reversible combination with the virus or that it alters host susceptibility.”

The results of several experiments carried out with two viruses, using a series of dilutions of an extract-inoculum mixture, are graphed in Figures 1 and 2.

The ratio of extract concentration to inoculum concentration does not alter throughout the series of dilutions, yet the reduction in lesion production induced by both inhibitors becomes progressively less as the mixture is diluted. In fact, the converging nature of the dilution curves indicates that it would theoretically be possible to overcome completely the effect of either inhibitor simply by sufficiently diluting a mixture of inoculum and extract. Thus, either the inhibiting constituents of the extracts are combined with the virus in some way that is readily dissociated by dilution, or their effect is on the host used for the infectivity tests and their tolerance of dilution is less than that of the virus.

(iv) *Effect of Inoculum Concentration.*—Caldwell (1935) showed that substances inhibiting infection by a virus could be distinguished from substances inactivating a virus because inhibitors have their greatest effect on concentrated

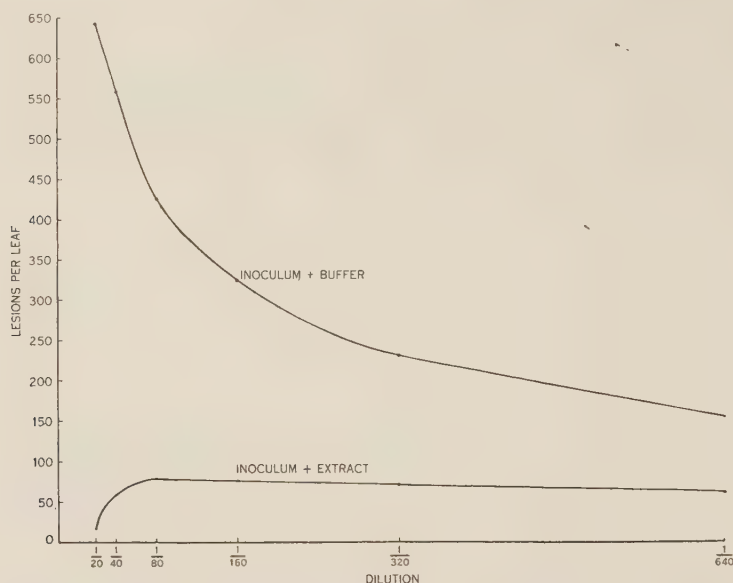


Fig. 1.—Effect of diluting a mixture of cucumber mosaic virus inoculum and cucumber embryo extract with buffer, on the infectivity of the mixture to cowpea.

TABLE 6

EFFECT OF CUCUMBER SEED EXTRACT ON LESION PRODUCTION BY COWPEA WHEN APPLIED BEFORE OR AFTER APPLICATION OF CUCUMBER MOSAIC VIRUS INOCULUM

Time of Application of Treatment Relative to Time of Application of Inoculum	Mean Number of Lesions per Half Leaf Following Treatment with:	
	Cucumber Seed Extract	Distilled Water
—48 Hr	1.1***	145
—24 Hr	0***	60
+24 Hr	191	194

*** Differences significant at $P = 0.001$.

inocula, whereas inactivators have their greatest effect on diluted inocula. Figures 3 and 4 show the results of experiments where constant amounts of extracts were added to a series of dilutions of cucumber and tobacco mosaic virus inocula. With both viruses the reduction in lesion numbers was consistently found to be greatest with concentrated inocula and it is concluded that the action of both seed extracts used is purely that of an inhibitor of virus infectivity.

(v) *Separate Applications of Extract and Inoculum.*—Two attempts were made to determine whether the action of the extracts was primarily on the host, or on the virus, by inoculating plants with the extract and the virus inoculum

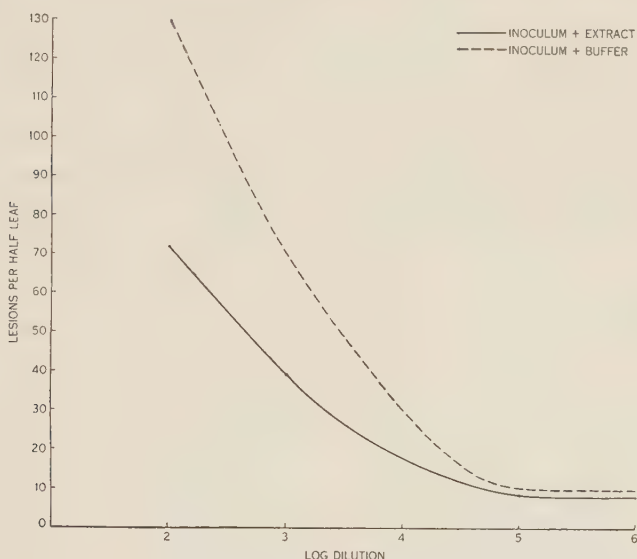


Fig. 2.—Effect of diluting a mixture of tobacco mosaic virus inoculum and tobacco seed extract with buffer, on the infectivity of the mixture to *N. glutinosa*.

separately. The first method was to treat the cowpea and *N. glutinosa* plants with the extract at various periods before and after inoculation with the virus inoculum.

TABLE 7

EFFECT OF TOBACCO SEED EXTRACT ON LESION PRODUCTION BY *N. GLUTINOSA* WHEN APPLIED BEFORE OR AFTER APPLICATION OF TOBACCO MOSAIC VIRUS INOCULUM

Time of Application of Treatment Relative to Time of Application of Inoculum	Mean Number of Lesions per Half Leaf Following Treatment with:	
	Tobacco Seed Extract	Distilled Water
—48 Hr	27.2**	63.5
—24 Hr	25.7**	73.3
Mixed with inoculum	3.8**	62.4
+24 Hr	56.0	58.6

** Differences significant at $P = 0.01$.

The results in Tables 6 and 7 show that both the seed extracts used were able to induce a highly significant reduction in the number of local lesions produced by their respective hosts when applied 1 or 2 days prior to the application

of the virus inoculum. From this it is concluded that both inhibitors are capable only of interfering with infection by their respective viruses. They are incapable of interfering with virus multiplication because both are incapable of producing any effect after infection has taken place.

The second method of separate application of the extracts and inocula was to apply the extract to the under surface of the leaves 24 hr before applying the virus inoculum to the upper surface.

TABLE 8
EFFECT OF UNDER-SURFACE PRE-TREATMENT WITH CUCUMBER EMBRYO EXTRACT ON LESION FORMATION BY CUCUMBER MOSAIC VIRUS ON QOWPEA

Leaf Under-Surface Pre-treated with:	Mean Number of Lesions per Leaf		
	Dilution of Extract		
	Undiluted	1/10	1/100
Extract	23	27*	32
Distilled water	35	33	29

* Difference significant at $P = 0.05$.

The results in Table 8 show that extracts of cucumber embryos can induce a significant reduction in the number of local lesions produced on cowpeas when applied in this manner. This also indicates that the effect of the cucumber embryo extract is to reduce the susceptibility of cowpeas to infection. In similar experiments carried out with tobacco mosaic virus a significant reduction in lesion numbers could not be induced by applying tobacco seed extracts to the under surface of *N. glutinosa* leaves. This is attributed firstly to the fact that tobacco seed extracts were always far less effective than cucumber embryo extracts, and secondly to the hairy nature of the under surface of *N. glutinosa* leaves that makes them most difficult to wet.

(c) Nature of the Inhibitors

Investigations were carried out to determine the nature of the constituents present in cucumber and tobacco seeds which are responsible for the inhibition.

The inhibitor from cucumber embryos was found to be heat-labile, non-dialysable, and can be precipitated from solution by alcohol or half-saturated ammonium sulphate. The inhibitor from tobacco seed was heat-stable, non-dialysable, and was precipitated from solution by 60 per cent. alcohol, but not by three-quarters-saturated ammonium sulphate. In tests kindly carried out by Dr. R. J. Swaby, of the Division of Soils, C.S.I.R.O., both substances were identified as proteins by their electrophoretic mobility and their staining reaction with bromphenol blue (Swaby, unpublished data). This finding is at variance

with the conclusion reached by Kausche that the virus-inhibiting substance present in tobacco seeds belongs to the amino-alcohol group, but he presented no evidence that either supports his claim or is at variance with that presented here.

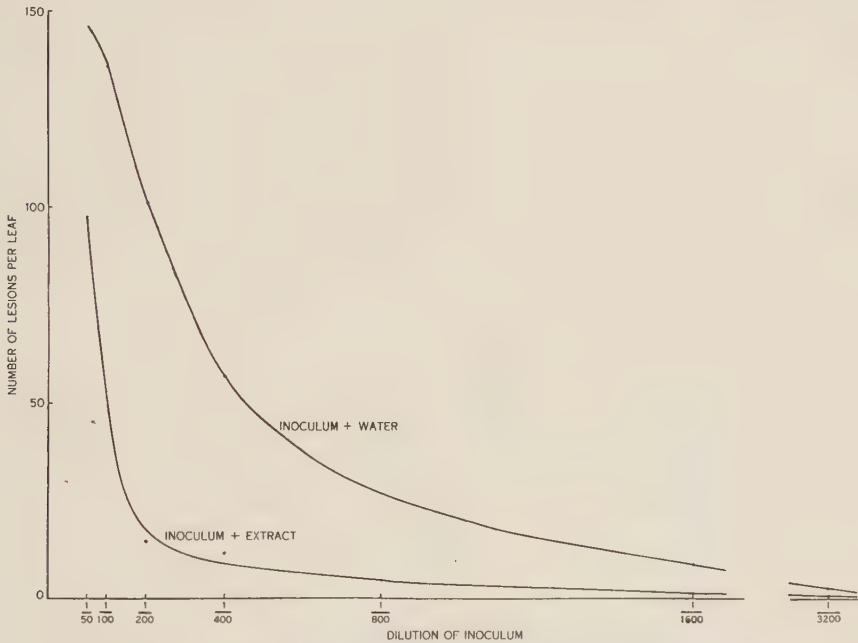


Fig. 3.—Effect of a constant amount of cucumber embryo extract on infectivity of several dilutions of cucumber mosaic virus to cowpea.

IV. DISCUSSION

The presence of an inhibitor of cucumber mosaic virus in the seeds of cucumber, and of tobacco mosaic virus in the seeds of tobacco, has been demonstrated. Six separate lines of evidence all indicate that the effect of these substances is, by some action on the host, to inhibit infection by the virus.

It is not thought possible that the presence of these inhibitors in the embryos of seeds can prevent the infection of embryos by plant viruses, for the inhibitors are also present in other tissues which undoubtedly can be infected. In studies carried out recently at Waite Institute on the distribution of viruses in the tissues of seeds, cucumber mosaic virus was found to be present in 92 per cent. of the testas, and only 4 per cent. of the embryos of mature seeds of wild cucumber, despite the fact that the inhibitor is present in approximately the same concentration in both tissues (see Table 1). Sill and Walker (1952) reported the presence of an inhibitor (possibly the same one as described here) of cucumber mosaic virus in all of the tissues, except the corolla, of cucumber plants. Yet no one would dispute the fact that the virus can infect these tissues, and multiply in them. It is far more likely that the action of the inhibitors is upon the local lesion host used for measuring virus infectivity. Its action could

be either to attach itself to the receptor sites in the cells at which virus multiplication is presumed to commence; or, as suggested by Bawden and Freeman (1952), for an inhibitor from the fungus *Trichothecium roseum* to "so alter the physiology of the host cells that they no longer support virus multiplication."

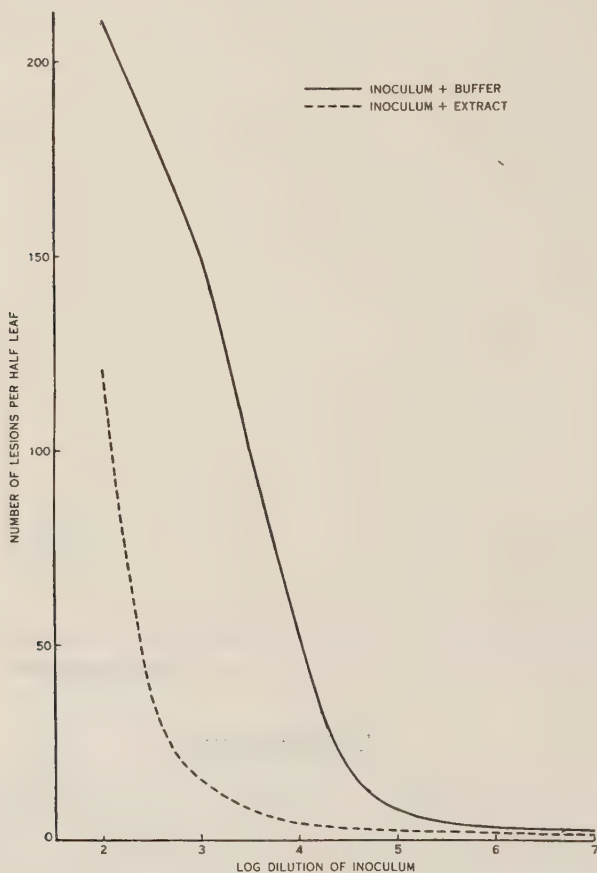


Fig. 4.—Effect of a constant amount of tobacco seed extract on infectivity of several dilutions of tobacco mosaic virus to *N. glutinosa*.

It might seem possible that inactivators could be present in the embryos of seeds in addition to the inhibitors described here, but if this were so it should be possible to demonstrate their presence by incubating seed extracts with viruses for some hours. Their presence would then be manifested by a greater decline in infectivity than would occur through normal aging of the inoculum. This was found not to be the case (see Tables 3 and 4).

It would seem that Duggar's theory that inactivators in seeds prevent the seed transmission of plant viruses must be abandoned as an explanation for the rarity of seed transmission of plant virus diseases, unless the inactivator is some transitory product of the metabolism of embryos. Such a transitory

product could be an enzyme or group of enzymes which breaks down virus particles, together with the other proteins of the endosperm, prior to their absorption by the developing embryo, and re-synthesis into embryo proteins. Inactivators of such a nature could inactivate viruses without ever accumulating to such an extent that their presence could be detectable by the usual infectivity techniques.

V. ACKNOWLEDGMENTS

The author wishes to acknowledge the helpful suggestions made by Dr. N. T. Flentje and Dr. R. J. Best on many aspects of the work; the help of Dr. R. J. Swaby in identifying the two proteins studied; of Dr. C. G. Hansford and Dr. N. T. Flentje in the preparation of the manuscript; of Mrs. I. Mathison for much help in the statistical design and analysis of experiments, and of Miss H. Lewis for able technical assistance.

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STRAINS OF *MYZUS PERSICAE* (SULZ.) ACTIVE AND INACTIVE WITH RESPECT TO VIRUS TRANSMISSION

By L. L. STUBBS*

[Manuscript received August 20, 1954]

Summary

The inconsistent transmission of a persistent yellows virus disease of spinach by its vector, *Myzus persicae*, is described.

A study was made of the infective ability of viviparously produced progeny of individual apterae, selected at random from a stock colony. It was found that individual cultures varied in their capacity to transmit the virus, and that selected cultures retained their infectivity characteristic in successive controlled experiments.

It is postulated that inactive insects may occur more frequently in vector species than is at present realized, and could account for much of the variability which characterizes virus-vector relationships.

I. INTRODUCTION

In experiments with a yellows virus of spinach, which is transmitted by the green peach aphid, *Myzus persicae* (Sulz.), inconsistent transmission of the virus occurred over a period of two years. As the inconsistent results could not be related to variations in virus source, or in test plants, an explanation was sought on the basis of differences between individual aphids.

The virus, which has not yet been identified with any known virus, and which will be described elsewhere, is of the persistent type. It is frequently associated with a strain of the cucumber mosaic virus in a complex infection of spinach. The yellows and cucumber mosaic viruses may be separated readily from a complex infection by applying the long and short feed techniques respectively, to the common vector, *M. persicae*.

II. METHODS

The virus was originally isolated from spinach, and was maintained on that host. Test plants were spinach seedlings in the first or second true leaf stage of development. One line of seed of the variety Nobel was used in all experiments, unless otherwise stated. Aphids were transferred to test plants with a camel's hair brush, and were caged on the plants by means of small celluloid cylinders with muslin tops. The long cotyledonary leaves of the spinach plants were held in a vertical position by loops of electrical fuse wire, thus permitting the use of small-diameter cages without disturbing the aphids, or injuring the plants.

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The plants were sprayed with "Systox" systemic insecticide at the end of the transmission feed, and held in an insect-proof cage for the duration of the experiment.

III. RESULTS

The variable transmission of the yellows virus by *M. persicae* first became apparent in experiments concerned with its persistency in that aphid. The results recorded in Experiment 1 are typical of those obtained from other experiments of this type.

(a) Experiment 1

In this experiment semi-mature non-infective apterae were given an acquisition feed of 24 hr, on one leaf of a spinach plant infected with the yellows virus. The aphids were carefully selected for uniformity of size from a vigorous colony on a healthy spinach plant. Eight groups, each of five aphids, were transferred serially, at 24-hr intervals, to healthy Nobel and U.S.D.A. 179590* spinach plants. The results and experimental details are recorded in Table 1.

TABLE 1
VARIABLE TRANSMISSION OF SPINACH YELLOWS VIRUS BY *M. PERSICAE*

Transfer No.	Transfer Date	Virus Persistence in <i>M. persicae</i> (days)	Infections Recorded							
			Nobel				U.S.D.A. 179590			
			1	2	3	4	1	2	3	4
1	13.v.53	0	—	—	+	—	—	+	—	—
2	14.v.53	1	—	—	+	—	—	+	—	—
3	15.v.53	2	—	—	+	+	—	+	—	—
4	16.v.53	3	—	—	+	—	—	+	+	—
5	18.v.53	5	—	—	+	—	+	—	—	—
6	19.v.53	6	—	+	+	—	—	+	+	—
7	20.v.53	7	—	—	+	—	—	+	—	—
8	21.v.53	8	—	—	+	—	—	—	—	—
9	22.v.53	9	—	—	+	—	—	—	—	—
10	25.v.53	12	—	—	—	—	—	—	—	—

(b) Experiment 2

The wide differences in infectivity between the groups of insects used in Experiment 1 suggested that some individuals were infective and others were not. The possibility also existed that many individual aphids did not possess the ability to transmit the spinach yellows virus. In order to gain evidence

* This accession was obtained through the courtesy of Dr. Paul G. Smith, University of California, U.S.A. In field trials at Burnley it was immune from infection with the cucumber mosaic virus but was highly sensitive to yellows.

for this hypothesis it was necessary to compare the infectivity of the progeny of a number of individual aphids. The mother aphids were selected as adult apterae from the stock non-infective colony used in Experiment 1. They were caged separately on each of the infected spinach plants listed in Table 2, which were selected from the above experiment.

When vigorous colonies had developed, groups of five adult apterae were transferred from each colony to healthy spinach seedlings, and serially transferred on three successive days to further test plants. The number of infections is recorded in Table 2.

Alate aphids were also examined from each colony, and their morphological characters were found to conform with those described for *M. persicae*.

TABLE 2
COMPARATIVE INFECTIVITY OF CLONAL CULTURES OF *M. PERSICAE*

Virus Source	Infections Recorded on Spinach				Vector Efficiency Rating
	Transfer Date				
	11.viii.53	12.viii.53	13.viii.53	14.viii.53	
Nobel T1/R3	+	+	+	+	100
Nobel T2/R3	+	+	+	+	100
Nobel T3/R4	+	—	+	—	50
Nobel T5/R3	+	—	—	—	25
Nobel T8/R3	+	+	—	—	50
Nobel T9/R3	+	+	+	+	100
U.S.D.A. 179590 T1/R2	+	+	—	—	50
U.S.D.A. 179590 T2/R2	+	+	+	—	75
U.S.D.A. 179590 T3/R2	+	+	—	—	50

(c) Experiment 3

In a further experiment the infectivity of aphids from Nobel colonies T1/R3 and T3/R4 was compared. Aphids from these colonies had shown high and low infectivity, respectively, in Experiment 2. In the experiment four groups of five alatae from each colony were placed on test seedlings and serially transferred on three successive days. The results (Table 3) showed that aphids from these colonies retained the same order of infectivity as exhibited in Experiment 2.

(d) Experiment 4

A final comparison of the infectivity of individual aphid colonies was made between colonies T1/R3, T2/R3 (high infectivity), T3/R4, and T5/R3 (low infectivity). Prior to the commencement of this experiment new colonies had been established on Nobel plants infected from the one virus source. Three

groups of five apterae from each colony were serially transferred to test seedlings as in Experiment 3. The infections recorded in Table 4 show that aphids from colonies T1/R3 and T2/R3 retained their infectivity, whereas those from colonies T3/R4 and T5/R3 were apparently unable to transmit.

It was the original intention of the author to maintain the above aphid colonies for further work with the spinach yellows virus, but they were inadvertently lost through fungal attack and an unfavourable environment during the summer period. However, an attempt will be made to select further active and inactive individuals from a field collection of *M. persicae* for the continuance of these studies.

TABLE 3
COMPARATIVE INFECTIVITY OF TWO CLONAL CULTURES OF *M. PERSICAE*

Transfer Date	Infections Recorded on Spinach							
	Colony T3/R4				Colony T1/R3			
	1	2	3	4	1	2	3	4
28.ix.53	—	—	—	—	—	+	+	—
29.ix.53	+	—	—	+	+	+	+	+
30.ix.53	—	—	—	—	+	—	+	+
1.x.53	—	+	—	—	—	—	+	+
Total infections	1	1	0	1	2	2	4	3

IV. DISCUSSION

The results obtained in the above experiments indicate that the aphid *Myzus persicae* is heterozygous for ability to transmit the spinach yellows virus. While these experiments do not prove conclusively the existence of this phenomenon, it would be difficult to suggest an alternative explanation.

In Experiment 2 the results could be explained on the basis that some virus source plants were a better source of virus than others. However, the fact that aphid cultures with high and low infectivity in this experiment retained their infectivity characteristic in succeeding experiments renders this explanation unlikely. Moreover, this explanation should not apply to Experiment 1, where all transfer aphids were given their acquisition feeding period on a single leaf of one infected spinach plant.

Individual variation in ability to transmit was first recognized by Storey (1932) in the leafhopper, *Cicadulina mbila*. This characteristic has since been recognized for other leafhopper species (Black 1943; Kunkel 1951; Maramorosch 1953), but the author is aware of only one attempt (Bawden and Kassanis

1947) to apply the findings of Storey to aphid vectors of plant viruses. These workers suggested "that occasional individual *M. persicae* or other potato aphids may be vectors although the bulk of such species are not" vectors of potato virus C. Their experiments with *M. persicae*, collected from many different sources and hosts, and other species of aphids, failed to support this hypothesis. In further transmission experiments with potato virus Y they reported wide

TABLE 4
COMPARATIVE INFECTIVITY OF FOUR CLONAL CULTURES OF *M. PERSICAE*

Transfer Date	Replication No.	Infections Recorded on Spinach			
		Colony T3/R4	Colony T1/R3	Colony T5/R3	Colony T2/R3
19.x.53	1	—	+	—	+
	2	—	+	—	—
	3	—	+	—	+
20.x.53	1	—	+	—	+
	2	—	+	—	+
	3	—	+	—	+
21.x.53	1	—	—	—	—
	2	—	+	—	—
	3	—	—	—	—
22.x.53	1	—	—	—	—
	2	—	+	—	+
	3	—	—	—	+
Total infections		0	8	0	7

differences in efficiency between a number of aphid species. They suggested, as the simplest explanation of their results, that the low infectivity of some species might relate to the relatively few active individuals in those species. Unfortunately, they did not attempt to verify their hypothesis by an experimental study of individual insects.

Aphids, because of their shorter life cycle, and their capacity for both parthenogenetic and sexual reproduction, are more suitable insects than leafhoppers for a study of inherent variation in transmission ability. It is strange therefore that the infective variability of these insects has received so little attention from plant virologists.

It would appear unlikely that individual variation in ability to transmit specific viruses is a characteristic shared by only three leafhoppers and one

aphid vector. It is possible, however, that inactive insects may be in the minority where an efficient virus vector relationship has been established, but they could predominate where a less efficient relationship exists.

An analysis of the work of Costa and Grant (1951) with *Aphis citricidus*, the vector of tristeza virus of citrus, raises some interesting possibilities. Their results showed that there was little difference in the infective ability of one, five, or 25 aphids (16, 10, and 21 per cent. transmission respectively), yet the transmission rate rose to 77 and 88 per cent. when 50 to 100, and 100 or more aphids were used. They conclude that "a minimum of 25 aphids per plant was necessary to obtain a high percentage of transmission." The aphids used by these workers were obtained from field collections from sweet orange.

Similarly, with strawberry virus 3, Prentice (1949) and Prentice and Woollcombe (1951) consistently recorded low transmission rates, when the acquisition and transmission feeding periods were together longer than the latent period.

These examples are quoted because the author has had experience with both viruses, and the apparently low efficiency of their insect vectors. In an experiment recently concluded, groups of 10 adult *Pentatrichopus fragaefolii*, bred on a single virus source plant infected only with virus 3, infected less than 50 per cent. of the *Fragaria vesca* test plants. These aphids were given a transmission feed of 5 days.

In experiments with a virus complex responsible for the decline of Ellendale mandarin (Stubbs 1952), groups of 20 aphids bred on infected plants have transmitted the complex, under optimum conditions, to 20-80 per cent. of inoculated seedlings. Cultures of both species of aphids used in the author's experiments were established from the newly born progeny of a number of insects. The relative efficiency of individual insects from both species is now being investigated by the method described above.

With increasing attention being given by virus workers to the elimination of variables in work on vector relationships (Miller 1952; Sylvester 1953), it is contended that the matter of vector efficiency should receive prior attention. It is conceivable that precise experiments with a vector population, established unwittingly from a single individual with low or moderate capacity to infect, would produce uniformly false data in relation to a specific virus. Conversely, work with a mixed population might introduce a greater variable than the sum total of variables eliminated by refined techniques.

As a preliminary, therefore, to insect transmission work with any persistent virus, it is suggested that the infective capacity of individual insects should be determined by a method similar to that described above.

V. ACKNOWLEDGMENTS

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The author is indebted to Mrs. A. Neboiss for assistance with this work.

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THE INFLUENCE OF NUTRITION ON THE WATER RELATIONS OF *SALMONELLA ORANIENBURG*

By J. H. B. CHRISTIAN*

[Manuscript received August 9, 1954]

Summary

The growth rates of *Salmonella oranienburg* have been determined in four different basal media in which the water activity (a_w) was controlled by addition of salts and by addition of sucrose. In three complex media the lower limit for growth was between 0.94 and 0.95 a_w but in a simple defined medium the limiting a_w for growth was between 0.96 and 0.97. When an amino acid mixture containing proline and methionine was added to salt-adjusted simple medium, growth occurred at 0.96 a_w . Subsequent addition of eight water-soluble vitamins extended the growth range to 0.95 a_w .

I. INTRODUCTION

In an earlier paper (Christian and Scott 1953) it was shown that various basal media supported the growth of several strains of salmonellae at water activities down to about 0.945. This lower limit for growth was substantially unaffected by variations in the concentration and type of nutrients in the medium, even though these media supported different rates of growth at the optimum a_w of 0.995. When the observations were extended to a simple medium of inorganic salts and glucose the expected decrease in growth rate at the optimum a_w was accompanied by a considerable reduction in the range of a_w 's permitting growth. This result indicated that growth at a_w 's of the order of 0.95 to 0.96 could only be realized when certain nutrients, absent from the simple medium, were provided. This paper reports the results of experiments which have defined some of these supplementary nutrients.

II. METHODS

The strain of *S. oranienburg* (206) used in all experiments was one of the 16 strains previously studied by Christian and Scott (1953). The methods for controlling a_w and for measuring rates of growth have been described by Scott (1953). The media used were quarter-strength brain heart broth (B.H., basal a_w 0.998); casamino acids-yeast extract-casitone (C.Y.C., 0.999 a_w); nutrient broth (N.B., 0.999 a_w); and a simple glucose-inorganic salts medium (G.S., 0.999 a_w) containing glucose (0.01m), $(\text{NH}_4)_2\text{SO}_4$ (0.005m), MgSO_4 (0.0005m), Na_2HPO_4 (0.014m), and KH_2PO_4 (0.006m). B.H. and C.Y.C. were described by Scott (1953). The solutes used to obtain the desired a_w 's were a mixture of NaCl, KCl, and Na_2SO_4 in the molal ratio 5 : 3 : 2 (Scott 1953) and

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sucrose. To test substances for stimulation of growth, 0.02-0.06 ml of aqueous stock solution was added aseptically to 10 ml of medium immediately prior to inoculation. Such additions altered the a_w of the medium by less than 0.001.

III. RESULTS

(a) Growth in Complex Media

The rates of growth of *S. oranienburg* in three complex media, B.H., N.B., and C.Y.C., in which the a_w was controlled by addition of the triple salt mixture and sucrose are shown in Figures 1 and 2 respectively. For salt-adjusted media in Figure 1, differences in rates of growth between the three media which were large at high a_w 's became smaller as the a_w was reduced to 0.96. Similarly in

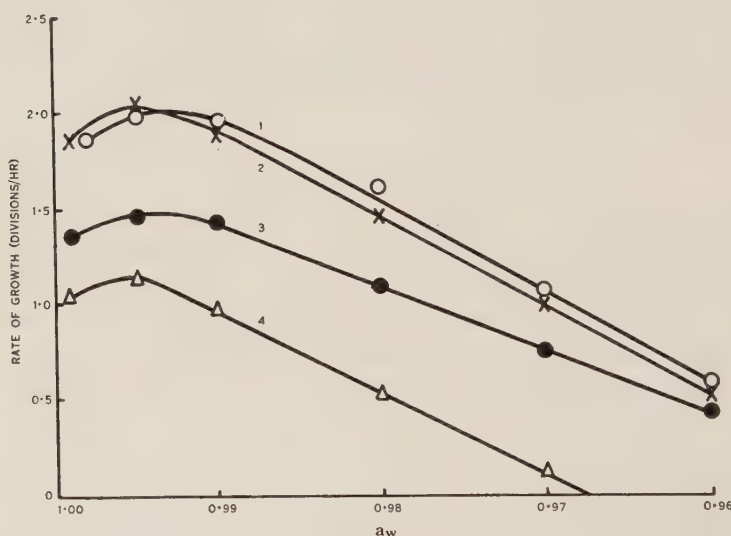


Fig. 1.—Relation between rate of growth and a_w for *S. oranienburg* in four media in which a_w was controlled with NaCl:KCl:Na₂SO₄ mixture in ratio of 5 : 3 : 2 moles. Curve 1: B.H. medium. Curve 2: N.B. medium. Curve 3: C.Y.C. medium. Curve 4: G.S. medium.

Figure 2 the curves for sucrose-adjusted media converged at about the same a_w . Although the absolute rates of growth at 0.96 a_w were about four times as high in salt-adjusted media as when sucrose was the principal solute, it has been shown previously (Christian and Scott 1953) that the lowest a_w 's permitting growth were virtually the same for both sucrose and the mixture of salts.

(b) Growth in G.S. Medium

In G.S. medium much lower rates of growth were observed at 0.995 a_w than in any of the three complex media and these rates decreased rapidly as the a_w was reduced to 0.97. The results were similar whether the a_w was controlled by the salts mixture or by sucrose as shown in Figures 1 and 2. No growth

occurred at 0.96 a_w within 28 days. Clearly, therefore, the range of a_w supporting growth in this medium is much smaller than in any of the three complex media.

(c) Growth in Supplemented G.S. Medium

The large differences in rates of growth and in osmotic tolerance of *S. oranienburg* in C.Y.C. and G.S. media were apparently due to differences in the nutrients supplied. C.Y.C. and G.S. media both contained inorganic salts and glucose, but the former was supplemented with yeast extract (Y.E.), casamino acids (C.A.), and casitone (C.T.). Therefore these three supplements were tested individually as growth stimulants in basal G.S. medium at 0.999 a_w and in salt-adjusted medium at 0.97 a_w .

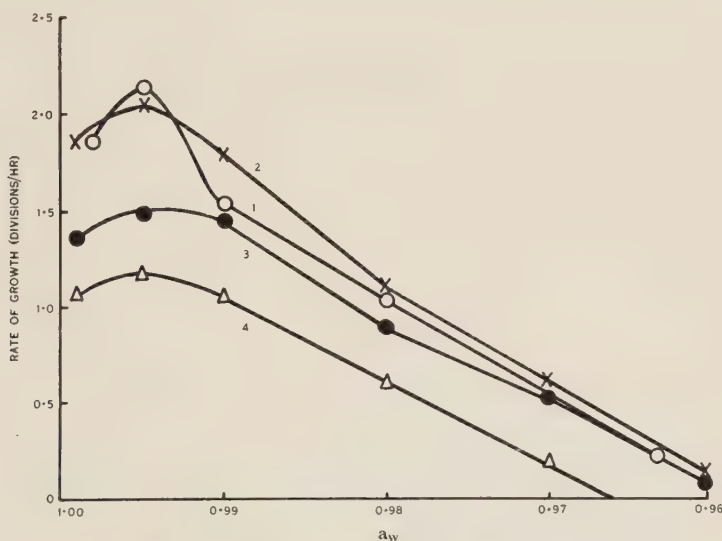


Fig. 2.—Relation between rate of growth of *S. oranienburg* in four media in which a_w was controlled with added sucrose. Curve 1: B.H. medium. Curve 2: N.B. medium. Curve 3: C.Y.C. medium. Curve 4: G.S. medium.

All three supplements increased the growth rate from 1.1 to 1.5 divisions/hr at 0.999 a_w at final concentrations of 500 mg/l. At 0.97 a_w Y.E. stimulated the growth rate from 0.1 to 0.95 divisions/hr and this initial rate was followed by two lower values of 0.58 and 0.35, as shown in Figure 3. C.A. and C.T. both increased the rate from 0.1 to 0.6 at 0.97 a_w , a result which was also obtained with a vitamin-free casamino acid preparation (V.F.C.A.). No stimulation was given by the ash of any of the supplements at either a_w or by an ether extract of Y.E. The ether-insoluble residue retained full activity. This suggested that stimulation was due to amino acids with the possibility of some additional substances present in Y.E. having an effect at 0.97 a_w .

A mixture of 22 amino acids duplicated the effect of V.F.C.A. but omission of proline reduced the stimulation to a very low level. Proline was therefore paired with each of the other 21 amino acids and four combinations were found

which produced greater stimulation of growth than did proline alone. When all of these effective amino acids, proline, methionine, serine, glutamic acid, and histidine, were tested together the rate of growth was very close to that observed with V.F.C.A. A summary of the results with various combinations of these five amino acids is given in Table 1. The final concentrations of these

TABLE 1
STIMULATION OF GROWTH OF *S. ORANIENBURG* BY AMINO ACIDS AT 30° C
Basal medium: G.S. medium adjusted with added salts to 0.970 a_w

Amino Acids Added*					Growth Rate (divisions/hr)
DL-Proline	DL-Serine	L-Glutamic acid	DL-Methionine	L-Histidine	
Basal medium only					0.10
+					0.27
+	+				0.45
+		+			0.35
+			+		0.52
+				+	0.37
+	+	+	+		0.59
+	+	+		+	0.42
+	+		+	+	0.54
+		+	+	+	0.53
	+	+	+	+	0.21
+	+	+	+	+	0.58
Vitamin-free casamino acids†					0.61

* Each amino acid was tested at a final concentration of 40 mg/l. The following did not give appreciable stimulation when coupled with proline: DL- α -alanine, DL- β -alanine, L-arginine, DL-aspartic acid, L-cysteine, glycine, L-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, DL-norleucine, DL-norvaline, DL-phenylalanine, DL-threonine, DL-tryptophan, L-tyrosine, DL-valine.

† Final concentration 500 mg/l.

+ Indicates addition of appropriate amino acid.

five amino acids when supplied by V.F.C.A. (7 per cent. total N) at 500 mg/l, based on an analysis of casein (McMeekin and Polis 1949), were: L-proline 29 mg/l, L-methionine 8 mg/l, L-serine 15 mg/l, L-glutamic acid 55 mg/l, L-histidine 8 mg/l. The growth rates observed when salt-adjusted G.S. medium was supplemented with the mixture of five amino acids at a_w 's from 0.999 to 0.96 are shown in Figure 4.

As Y.E. had stimulated the growth rate to 0.9 divisions/hr in salt-adjusted G.S. medium it was possible that some of the B vitamins were involved. A mixture of eight vitamins was added to the salt-adjusted G.S. medium to give the following final concentrations: thiamin, 0.8 mg/l; riboflavin, 0.2 mg/l; biotin, 4 μ g/l; folic acid, 8 μ g/l; pyridoxine, 0.8 mg/l; Ca pantothenate, 0.8 mg/l;

nicotinic acid, 2 mg/l; *p*-aminobenzoic acid, 2 mg/l. No response was obtained at any a_w in the absence of the five amino acids but in their presence stimulation occurred at lower, but not at higher a_w 's, as shown in Figure 4. Tests of the individual vitamins and of combinations of seven in salt-adjusted G.S. medium at 0.96 a_w with added amino acids all showed some stimulation and it was not possible to assign the effects of the mixture to any particular vitamin or vitamins.

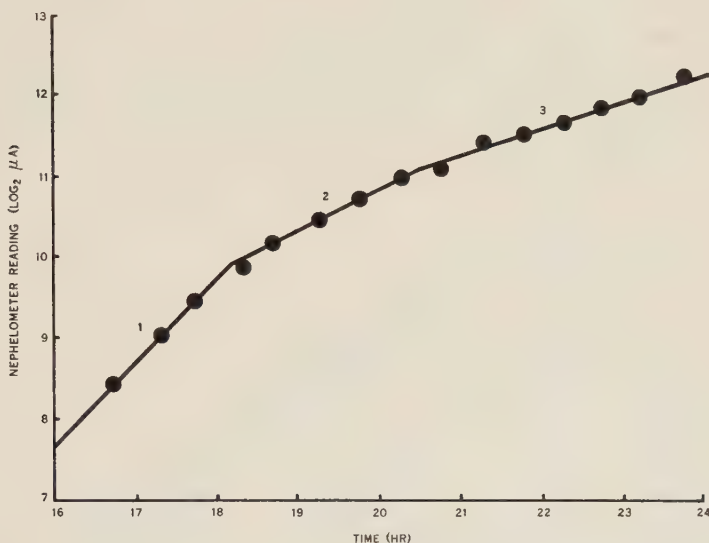


Fig. 3.—Typical growth curve for *S. oranienburg* in G.S. medium adjusted to 0.970 a_w with triple salt mixture and supplemented with 500 mg/l yeast extract. Rates: Curve 1: 0.95 divisions/hr. Curve 2: 0.58 divisions/hr. Curve 3: 0.35 divisions/hr.

A purine-pyrimidine mixture of adenine, guanine, uracil, and xanthine each at a final concentration of 2 mg/l did not affect the growth rate in salt-adjusted G.S. media containing five amino acids and eight vitamins at any a_w .

Stimulation by V.F.C.A. in G.S. medium adjusted to 0.97 a_w with sucrose was of the same order as in salt-adjusted medium. However, the addition of Y.E. to sucrose-adjusted medium produced only a small increase in growth rate from 0.18 to 0.36 divisions/hr, and was not investigated further.

(d) Minimum a_w 's for Growth

The lower limits of a_w supporting growth of *S. oranienburg* within 28 days at 30°C are given in Table 2. Irrespective of the method employed to control a_w , all complex media supported growth at 0.95 a_w , while in G.S. medium growth was recorded at 0.97 but not at 0.96 a_w . In salt-adjusted G.S. medium supplementation with five amino acids permitted growth at 0.96 a_w and with the subsequent addition of eight vitamins growth occurred at 0.95 a_w .

(e) Training Experiments

The possibility of training salmonellae to grow at increased rates under somewhat difficult osmotic conditions has been examined in two experiments. In C.Y.C. medium in which complex nutrients were provided, no evidence of increased growth rates was found during 20 transfers at $0.96 a_w$ using the triple salt mixture to control a_w . Growth rates made on each alternate transfer showed no significant regression with the number of transfers, the mean rate being 0.38 divisions/hr and the range for all values 0.35-0.40. In G.S. medium to which no complex nutrients were added, a similar experiment at $0.975 a_w$ showed slight evidence of a progressive increase in growth rate, the positive regression between rate of growth and number of transfers being significant ($P < 0.05$). The absolute increase in growth rate was, however, small, all values being within the range of 0.28-0.33 divisions/hr.

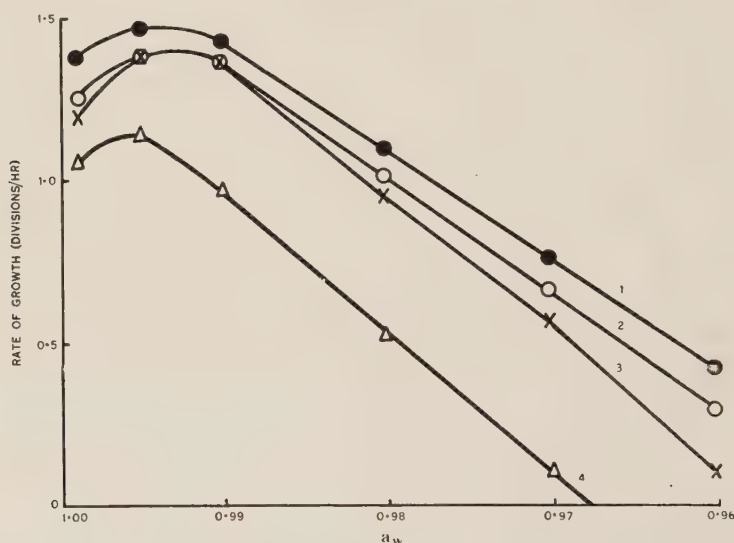


Fig. 4.—Relation between rate of growth and a_w for *S. oranienburg* in four media in which a_w was controlled with NaCl:KCl:Na₂SO₄ mixture in ratio of 5:3:2 moles. Curve 1: C.Y.C. medium. Curve 2: G.S. medium + five amino acids + eight vitamins. Curve 3: G.S. medium + five amino acids. Curve 4: G.S. medium.

IV. DISCUSSION

These results provide another example of the influence of the environment on the nutritional requirements of microorganisms. In respect of oxygen tension, Richardson (1936) has shown that the requirement of *Staphylococcus aureus* for uracil is limited to anaerobic conditions while Knight (1936) has also shown that some strains of *Salmonella* require tryptophan for anaerobic but not for aerobic growth. The effect of temperature has been demonstrated by Hills and Spurr (1952) for *Pasteurella pestis* which required several substances at 36° but not at 28°C. Similarly Beadle and Tatum (1945) have described the pro-

duction of *Neurospora* mutants whose nutritional needs are a function of temperature. Instances in which the osmotic environment modified the nutritional pattern do not, however, appear to have been reported previously.

The amino acid and vitamin requirements for growth of a number of exacting strains of *Salmonella* have been described by Knight (1936) and by Johnson and Rettger (1943) but these have little in common with the nutritional pattern of *S. oranienburg* under adverse osmotic conditions. In the pre-

TABLE 2

MINIMUM a_w 's FOR GROWTH OF *S. ORANIENBURG* IN VARIOUS LIQUID MEDIA AT 30°C AFTER 28 DAYS' INCUBATION

Basal Medium	Solutes Added	a_w	
		Growth	No Growth
B.H.	Salts mixture	0.95	0.94
B.H.	Sucrose	0.95	0.94
N.B.	Salts mixture	0.95	0.94
N.B.	Sucrose	0.95	0.94
C.Y.C.	Salts mixture	0.95	0.94
C.Y.C.	Sucrose	0.95	0.94
G.S.	Salts mixture	0.97	0.96
G.S.	Sucrose	0.97	0.96
G.S. + five amino acids	Salts mixture	0.96	0.95
G.S. + five amino acids + eight vitamins	Salts mixture	0.95	0.94

sent investigations, vitamins were essential for growth at 0.95 a_w and amino acids for growth at a_w 's below 0.97. However, while amino acids stimulated growth appreciably at high a_w 's the vitamins did not. It is of note that the vitamins were not effective in the absence of amino acids.

The observation that sucrose is more inhibitory than the salts mixture in complex media at a_w 's below the optimum without affecting the lower limit for growth has not been investigated. Obviously high sucrose concentrations have viscosities which might be expected to reduce the diffusion rates of nutrients in the solution. However, there is no evidence that this is important. Indeed, observations of a brewers' strain of *Saccharomyces cerevisiae* in complex medium showed the reverse effect—rates were lower in the salt-adjusted medium.

The results of the training experiments suggest that mutation to more osmotically tolerant forms will not be simple. This might be expected from the nutritional studies, which showed the need for a number of substances for growth at low a_w 's. The supplementary substances which increase the rate of growth of *S. oranienburg* at low a_w 's are also responsible for increasing the range of a_w 's over which growth will occur. Analogous studies with other organisms are needed to decide whether or not these nutrients have a general importance in the water economy of bacteria.

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STUDIES ON CHITIN

II. REACTION of *N*-ACETYL-D-GLUCOSAMINE WITH α -AMINO ACIDS, PEPTIDES, AND PROTEINS

By R. H. HACKMAN*

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Summary

The reaction of *N*-acetyl-D-glucosamine with α -amino acids, peptides, and proteins has been investigated. The course of the reaction has been followed by methods which involved the use of paper partition chromatography, polarimetric measurements, measurement of pH depression, and equilibrium dialysis. It is considered that products of the Schiff base (or azomethine) type are formed but the combination is unstable and is split even in the biological pH range. No reaction occurs under acidic conditions but under alkaline conditions the amount of reaction increases with the pH. At a given pH the percentage combination depends upon the equilibrium constant of the reaction and upon the dissociation constant of the α -amino nitrogen, as this determines the initial concentration of the reagent. Differences in the degrees of ionization are more important in determining the percentage combination than are the differences in the equilibrium constants. The reaction is of a similar type to that which occurs between glucose and α -amino acids or peptides. Tyrosine, as well as free α -amino groups, appears to play an important part in the binding of *N*-acetyl-D-glucosamine by insect cuticular proteins. 2-Acetamido-2-deoxy-*N'*-D-glucosylglycine ethyl ester has been synthesized from *N*-acetyl-D-glucosamine and glycine ethyl ester and its properties have been investigated.

I. INTRODUCTION

The arthropod cuticle is generally thought to contain a glycoprotein resulting from the combination of chitin and protein. The nature of this association is not clear and it is not known whether such an association is useful in the insect's physiology or whether it is the result of the mechanism of chitin synthesis. Although the nature of the bonding is unknown a number of conclusions can be drawn from published papers.

The data obtained from X-ray diffraction (Fraenkel and Rudall 1947) and electron microscope (Richards and Korda 1948) studies on chitin micelles have been interpreted as indicating the presence of a weak bonding between chitin and protein in the soft cuticles of insects. This idea of a weak bonding is supported by the fact that the major part of the protein in soft cuticles can be separated from the chitin by relatively gentle methods, e.g. extraction with water and buffer solutions (Hackman 1953*a*, 1953*b*; Trim 1941). On the other hand Trim (1941) has shown that the puparia of blowflies (*Sarcophaga falcu-*

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lata) can be dispersed in lithium thiocyanate at 170°C and reprecipitated without effecting a separation of the chitin and protein components. This suggests that in blowfly puparia there exists quite a strong bond between the chitin and protein. It can be concluded from these papers that, in insect cuticle, there is probably some chitin-protein bonding and that the bonding in hardened cuticle is much stronger than that in soft cuticles.

Although no work has been published on the reaction between amino acids or peptides and *N*-acetyl-D-glucosamine (the recurring chemical unit of chitin) reference has been made to the reaction of *N*-acetyl-D-glucosamine with "dry" casein (pH 6.3, 37°C, and 70 per cent. R.H.) (Lea and Rhodes 1952). The reaction between the protein and *N*-acetyl-D-glucosamine was very slow when compared with the reaction of other carbohydrates, e.g. glucose and "dry" casein under similar conditions. The authors suggested that the slowness of the reaction may be due to the acetamido group inhibiting reaction at the terminal carbonyl group. In contrast to this lack of information on the reaction between amino acids and *N*-acetyl-D-glucosamine are the extensive investigations which have been carried out on the mechanism of the reaction between amino acids and aldose sugars such as glucose.

II. EXPERIMENTAL

(a) Reagents

The *N*-acetyl-D-glucosamine was a commercial preparation which was crystallized from a mixture of water, ethanol, and ether. It had m.p. 205°C (decomp.) and $[\alpha]_D^{24.9} 40.4^\circ$ (*c*, 8.98 in water).

The amino acids and peptides used were shown to be single identities by paper chromatography and before use they were dried to constant weight.

The protein used was the water soluble protein extracted from larval cuticles of *Diaphonia dorsalis* Don. (Coleoptera). For a description of the method of extraction see Hackman (1953a). The protein contained 13.9 per cent. N (micro-Kjeldahl).

(b) Paper Partition Chromatography

Lysine monohydrochloride (900 mg) and *N*-acetyl-D-glucosamine (2.21 g) were dissolved in a small quantity of water and the pH of the solution adjusted to approx. 10 with 7.5N aqueous sodium hydroxide. The total volume of the solution was 4.5 ml, which gave 1M lysine and 2M *N*-acetyl-D-glucosamine. The solution was kept at 37°C for 6 days, when the pH of the solution had dropped considerably and the formerly colourless solution had become reddish brown. The solution was diluted 75 times with water and subjected to paper partition chromatography using *n*-butanol-acetic acid-water (76.6-17 v/v) as the solvent and Whatman No. 1 filter paper at a temperature of 25°C. Besides spots corresponding to lysine and *N*-acetyl-D-glucosamine there appeared a spot between the origin and lysine which was detected with ninhydrin.

In a similar manner a solution containing glycine (1M) and *N*-acetyl-D-glucosamine (2M) at an initial pH of 9.5 when kept at 37°C for 7 days gave, after dilution with water and paper partition chromatography, a spot reacting with ninhydrin between the origin and the spot given by glycine. As before the final pH of the solution was less than 9.5 and the solution had become dark in colour.

The reaction products detected above are not stable on dilution with water. If any appreciable time elapses between diluting the solution with water and applying the diluted solution to the paper no additional spot is detected.

(c) *Synthesis of 2-Acetamido-2-deoxy-N'-D-glucosylglycine Ethyl Ester*

Freshly prepared glycine ethyl ester (2.8 g) was added to a suspension of anhydrous *N*-acetyl-D-glucosamine (6.0 g) (molar ratio 1 : 1) in magnesium-dried ethanol (15 ml). The mixture was boiled under reflux on a steam-bath for 5 hr and protected from moisture by a calcium chloride tube. Unreacted *N*-acetyl-D-glucosamine (2.07 g) was separated from the cooled solution by filtration and washed with absolute ethanol. The combined filtrates were concentrated to c. 5 ml, acetone (15 ml) added, and kept in a refrigerator overnight. The crystals which separated were collected by filtration, pressed well on the Büchner funnel, washed with ethanol-acetone (1:3; 5 ml), and dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. Yield: 3.1 g, m.p. 114°C. Repeated crystallization from absolute ethanol raised the m.p. to 141°C. (Found: C, 46.8; H, 7.1; N, 9.1 %. Calc. for $C_{12}H_{22}O_7N_2$: C, 47.0; H, 7.2; N, 9.1%).

The stability of 2-acetamido-2-deoxy-*N'*-D-glucosylglycine ethyl ester was determined by measuring the change with time in the pH of 0.001M solutions in water and 0.005M solutions in 0.005N aqueous hydrochloric acid. For purposes of comparison the change with time in the pH of an equimolar (0.001M) mixture of *N*-acetyl-D-glucosamine and glycine ethyl ester was determined.

(d) *Stability of N-acetyl-D-glucosamine at Different Temperatures and pH*

Since *N*-acetyl-D-glucosamine is unstable under alkaline conditions the relative amounts of degradation in 0.2M aqueous solutions at pH 8.0, 9.0, and 9.55 (buffers as for polarimetric experiments—see(e)) at 25°C and pH 9.0 at 37°C were determined as follows.

N-acetyl-D-glucosamine solution (1 ml) was diluted with glacial acetic acid, dimethylaminobenzaldehyde solution (1 ml of a 2 per cent. solution in glacial acetic acid containing 5 per cent. 10N hydrochloric acid) added, and the volume made up to 10 ml with glacial acetic acid. After 45 min the optical density of the solution was measured at 560 m μ . Water (1 ml) was used for preparation of the blank solution. Measurements were made of the amount of colour developed in buffer solution of *N*-acetyl-D-glucosamine which had been kept for varying periods up to 24 hr.

(e) Polarimetric Measurements

Glycine and glycylglycine were chosen for study by the polarimetric method because they are optically inactive and so the complications due to the change in the rotatory power of the amino acid or peptide itself were eliminated. The reactions were carried out at a constant pH, i.e. in buffer solution.

The glycine or glycylglycine was dissolved in water and 1N aqueous sodium hydroxide added to bring the solution to the desired pH, and then the solution was made up to volume with buffer (for pH 9.0 and 9.5 a carbonate-bicarbonate buffer (Delory and King 1945) and for pH 8.0 a veronal buffer (Michaelis 1930)). Final concentrations of glycine and glycylglycine were 0.4M. The *N*-acetyl-D-glucosamine (0.4M) was made up in buffer. Equal volumes of the *N*-acetyl-D-glucosamine and glycine or glycylglycine were mixed and for the purposes of control an equal volume of each was mixed with an equal volume of buffer. The reaction was allowed to proceed at the various pH values and various temperatures until equilibrium was reached, the progress of the reaction being followed by the decrease in optical rotation of the solution. In most experiments the molar ratio of the reactants was 1:1 but in one experiment this was altered to 1 mole glycine to 0.424 mole *N*-acetyl-D-glucosamine. As a check on the method, the amount of unreacted *N*-acetyl-D-glucosamine was determined iodometrically using the method for glucose as published by Dykins and Englis (1931). The method of Morgan and Elson (1934) could not be used to estimate the *N*-acetyl-D-glucosamine because under the hot alkaline conditions used further reaction would take place between the *N*-acetyl-D-glucosamine and the amino acid or peptide.

(f) Measurement of pH Depression

The amino acid or peptide was dissolved in water, the solution brought to pH 9.0 by the addition of 1N aqueous potassium hydroxide, water added to bring the solution to the desired concentration, and the pH again checked. In this manner 0.2M solutions of glycine, L-tyrosine, and L-lysine and 0.1M solutions of glycylglycine and L-leucylglycine were prepared. The *N*-acetyl-D-glucosamine (2M) was dissolved in water. Equal volumes of the *N*-acetyl-D-glucosamine and amino acid or peptide solutions were mixed. The progress of the reaction was followed by measuring the difference between the pH of the carbohydrate-amino acid or peptide solution and the pH of the amino acid or peptide solution after it had been diluted once with water. The pH of a 2M aqueous *N*-acetyl-D-glucosamine solution remained constant. The experiments with amino acids were conducted at 18°C, those with peptides at 22°C. All measurements were made on a Cambridge pH meter.

(g) Iodination of Insect Cuticular Protein

Larval cuticular protein (200 mg) was dissolved in aqueous ammonia (sp.gr. 0.88; 2 ml) and the solution cooled in an ice-bath. An aqueous solution of iodine in potassium iodide (2.8N; 0.2 ml) was added gradually to the vigorously stirred protein solution maintained at 0°C. After 1 hr water was

added, the solution neutralized with glacial acetic acid, and the precipitate collected by centrifugation. The precipitate was suspended in a small volume of water and dialysed against running water for 3 days and against distilled water for 2 days. The precipitate and solution was dried to constant weight at room temperature *in vacuo* over phosphorus pentoxide and potassium hydroxide. Yield: 165 mg. (Found: N, 12.0 % (micro-Kjeldahl)).

(h) *Acetylation of Insect Cuticular Protein*

Larval cuticular protein (200 mg) was suspended in half-saturated aqueous sodium acetate (4 ml). Acetic anhydride (0.3 ml) was added during 1 hr to the vigorously stirred protein suspension maintained at 0°C. The suspension was diluted with water and dialysed against running water for 3 days and against distilled water for 2 days. The precipitate and solution was dried to constant weight at room temperature *in vacuo* over phosphorus pentoxide and potassium hydroxide. Yield: 154 mg. (Found: N, 13.7 % (micro-Kjeldahl)).

(i) *Interaction of N-Acetyl-D-glucosamine with Insect Cuticular Protein*

Cellophane bags were prepared from commercial sausage casing (24/32) and filled with 10 ml of protein solution (0.19 per cent. in carbonate-bicarbonate buffer (*vide supra*) pH 8.77 at 37°C). Three drops of toluene were added as a preservative. The bag was immersed in 20 ml of a solution of *N*-acetyl-D-glucosamine (concentration range 0.233 to 1.953 mM) in the same buffer contained in a tube 1.125 in. internal diameter. Five drops of toluene were added as a preservative. The tube was closed with a rubber stopper and kept at 37°C for 3 days, by which time equilibrium had been attained. The external solution was analysed for *N*-acetyl-D-glucosamine by a slightly modified form of the method of Morgan and Elson (1934). To develop maximum colour 0.3 ml 0.5N aqueous sodium carbonate solution was added for each ml of buffer solution and the colour intensity was read after 100 min instead of 45 min. For each concentration of *N*-acetyl-D-glucosamine a control tube was prepared which differed from the test tube in that the cellophane bag contained 10 ml buffer solution and not the protein solution. Analysis of the internal and external solutions of the control tube for *N*-acetyl-D-glucosamine ensured that equilibrium had been reached. From the difference in concentration of *N*-acetyl-D-glucosamine in the external solutions of the control and test-tubes the amount of carbohydrate taken up by the protein could be calculated. By this method of using control and test-tubes it was possible to correct automatically for any adsorption of *N*-acetyl-D-glucosamine by the cellophane bag. No adsorption was detected. All experiments were conducted in duplicate.

This same experiment was repeated using the iodinated and acetylated proteins, using an equivalent protein concentration.

III. RESULTS AND DISCUSSION

From the filter paper partition chromatographic study of the reaction between amino acids and *N*-acetyl-D-glucosamine it is clear that an unstable condensation product is formed with glycine and lysine. The results obtained,

other than the instability of the compounds formed, are similar to those obtained by Gottschalk and Partridge (1950) for the reaction between glucose and lysine, and suggest that a similar type of reaction has occurred, namely, that initially compounds of the Schiff base (or isomeric *N*-glycoside) type are formed. The decrease in the pH of the solution indicates that reaction has occurred at the basic group of the amino acid. It is to be expected that a quantitative measure of the extent of the reaction can be obtained from the magnitude of the decrease in pH.

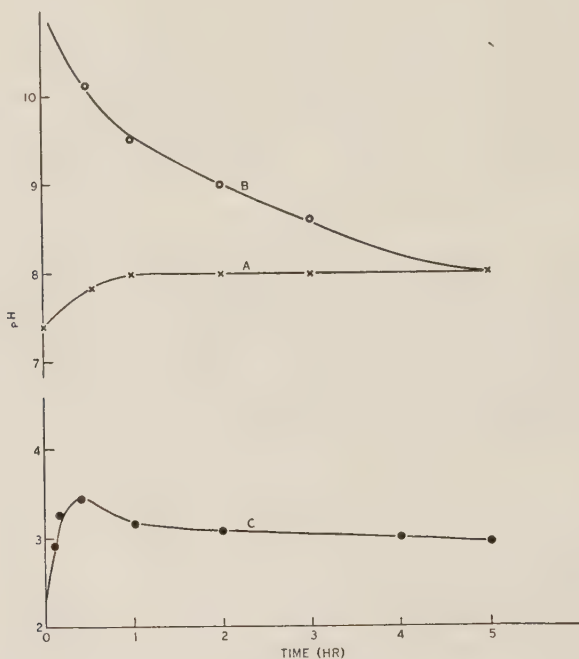


Fig. 1.—Stability (change in pH) at 23°C of 2-acetamido-2-deoxy-*N'*-D-glucosylglycine ethyl ester in water (0.001M; curve A) and to aqueous hydrochloric acid (0.005M in 0.005N acid; curve C). Curve B records the change in pH of a dilute aqueous solution of an equimolar (0.001M) mixture of *N*-acetyl-D-glucosamine and glycine ethyl ester.

All attempts to isolate condensation products from mixtures of amino acids and *N*-acetyl-D-glucosamine in aqueous or in anhydrous alcoholic solutions were unsuccessful. However, 2-acetamido-2-deoxy-*N'*-D-glucosylglycine ethyl ester was prepared from glycine ethyl ester and *N*-acetyl-D-glucosamine in anhydrous ethanol. The compound had zero optical activity when dissolved in anhydrous ethanol. As is shown in Figure 1, a dilute aqueous solution (0.001M) of this *N*-glycoside undergoes hydrolysis at room temperature (23°C), the pH rising from 7.40 to 8.00 in 2 hr (curve A). The pH of a dilute aqueous solution of an equimolar (0.001M) mixture of *N*-acetyl-D-glucosamine and glycine ethyl

ester gradually falls from 10.34 to 8.00 during 5 hr (curve *B*). These two pH-time curves finally merge (Fig. 1) and these results are those which would have been predicted on the assumption that the *N*-glycoside hydrolyses partially to *N*-acetyl-D-glucosamine and glycine ethyl ester. Towards mineral acid the *N*-glycoside is very unstable at 23°C, as is shown by curve *C* in Figure 1. Not

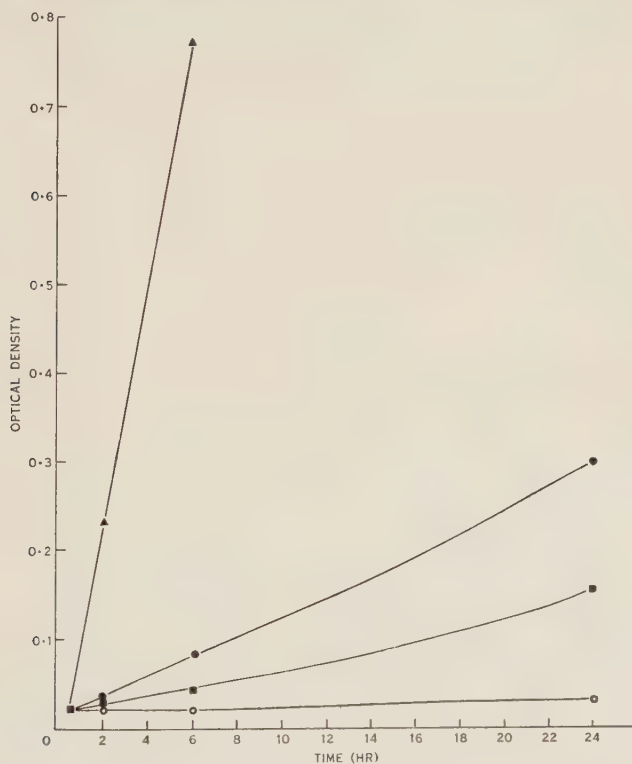


Fig. 2.—Stability of *N*-acetyl-D-glucosamine (0.2M) at different temperatures and pH. The optical density of the colour formed with Ehrlich's reagent has been plotted against time.

O — O, pH 8.0, 25°C.
 ■ — ■, pH 9.0, 25°C.
 ● — ●, pH 9.5, 25°C.
 ▲ — ▲, pH 9.0, 37°C.

only is the *N*-glycosidic link hydrolysed but the ester grouping also undergoes hydrolysis. This hydrolytic behaviour shows that only a small amount of the *N*-glycoside could be formed by the interaction of *N*-acetyl-D-glucosamine and glycine ethyl ester in neutral aqueous solutions while no complex formation would be expected under acidic conditions. This conclusion is in general agreement with what is known about the interaction of amino acids and aldose sugars such as glucose.

In view of the failure to isolate condensation products from mixtures of amino acids and *N*-acetyl-D-glucosamine the course of the reaction in aqueous

solution was investigated further by means of polarimetric measurements and measurements of pH depression. Two types of reaction conditions were chosen, one at constant pH, the other in which the pH was not kept constant. As is shown in Figure 2, *N*-acetyl-D-glucosamine is not stable under alkaline conditions. With increase in pH and increase in time the amount of colour formed with Ehrlich's reagent increases. Since the nature of the degradation is unknown (cf. Aminoff, Morgan, and Watkins 1952) it is not possible to determine

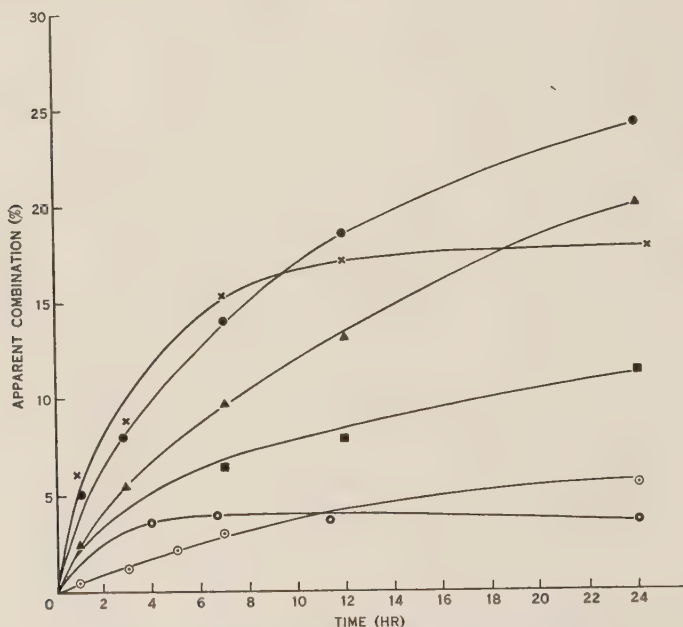


Fig. 3.—Apparent combination of *N*-acetyl-D-glucosamine (0.2M) with glycine and glycylglycine as determined by polarimetric measurements.

- — ○, Glycine 0.2M, pH 8.0, 25°C.
- — ■, Glycine 0.2M, pH 9.0, 25°C.
- ▲ — ▲, Glycine 0.2M, pH 9.5, 25°C.
- × — ×, Glycine 0.2M, pH 9.0, 37°C.
- — ●, Glycine 0.472M, pH 9.0, 25°C.
- ⊙ — ⊙, Glycylglycine 0.2M, pH 9.0, 25°C.

the absolute rate of decomposition but only the relative rate. Consequently the results are expressed in the form optical density *versus* time. It was not possible to prepare a true calibration curve because, on heating *N*-acetyl-D-glucosamine in aqueous sodium carbonate solution, the compound responsible for the formation of colour with Ehrlich's reagent is both formed and destroyed. Consequently the colour intensities as recorded in a calibration curve are probably lower than they would be if no destruction of the compound occurred in hot alkaline solution. However, by use of such a calibration curve it is possible to calculate that after 24 hr at 25°C the amount of degradation at pH 8.0 is

less than 0.03 per cent., at pH 9.0 less than 0.15 per cent., at pH 9.5 less than 0.30 per cent., and at 37°C and pH 9.0 less than 2.28 per cent. In view of the instability of *N*-acetyl-D-glucosamine in alkaline solution experimental conditions were confined to the pH range 8.9-5.

The progress of the reaction between 0.2M *N*-acetyl-D-glucosamine and 0.2M glycine or glycyglycine at constant pH was followed by determining the change with time in the optical activity of the solution. The results are given in Figure 3. Calculation of the percentage combination and equilibrium constants were made on the assumption that the combination is in an equimole-

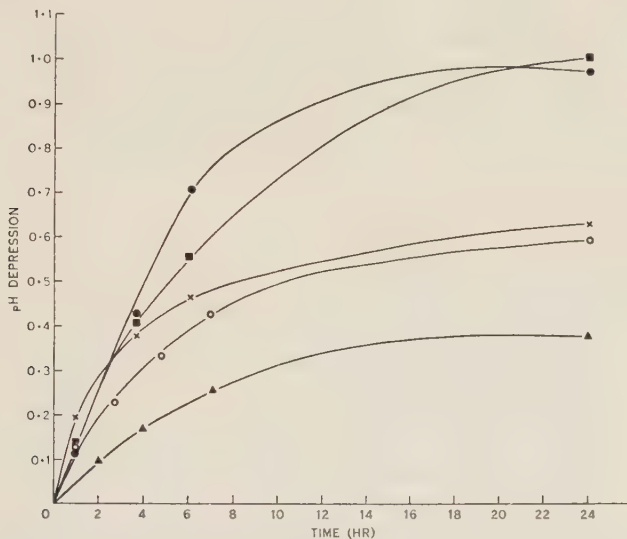


Fig. 4.—Increase of pH depression with time in the interaction of *N*-acetyl-D-glucosamine (1M) with amino acids (0.1M) and peptides (0.05M).

- — ■, Glycine, initial pH 9.0 and 18°C.
- — ●, L-Tyrosine, initial pH 9.0 and 18°C.
- × — ×, L-Lysine, initial pH 9.0 and 18°C.
- — ○, L-Leucylglycine, initial pH 8.68 and 22°C.
- ▲ — ▲, Glycyglycine, initial pH 9.0 and 22°C.

cular ratio and that the compound formed is of negligible rotation so that the decrease in rotation is equal to the extent of combination. This assumption may be open to criticism but the following percentage combinations obtained by iodometric titration support the assumption as a working approximation. Percentage combination found for glycine (after 24 hr at 25°C) by iodometric titration: pH 8.0, 3.1 per cent.; pH 9.0, 9.7 per cent; and pH 9.5, 17.0 per cent. As was to be expected, an increase in temperature increased the rate of the reaction whilst altering the ratio of *N*-acetyl-D-glucosamine to glycine shifted the equilibrium in accordance with the law of mass action. The equilibrium constant for the system containing *N*-acetyl-D-glucosamine and glycine at pH 8.0 was found to be 0.14×10^{-2} ; at pH 9.0, 2.7×10^{-2} ; and at pH 9.5,

4.9×10^{-2} ; and for glycylglycine at pH 9.0, 0.92×10^{-2} . From Figure 3 it is clear that equilibrium was reached in 24 hr and that the rate of reaction increases considerably as the pH increases from 8 to 9.5. A similar conclusion has been reached by workers investigating the reaction of aldoses, e.g. glucose or xylose with amino acids.

The polarimetric method could not be used successfully to investigate the nature of the reaction with amino acids other than glycine because of the complications introduced by the optical activity of the amino acids. The method involving measurement of pH depression (cf. Frankel and Katchalsky 1941), however, is quite applicable. The theoretical considerations of Frankel and Katchalsky contain a number of assumptions, but the formulae derived by them do represent the experimental conditions with some degree of accuracy and are quite adequate for the present study. The equilibrium constants and the percentage combination have been calculated from the formulae

$$\text{antilog } \Delta \text{pH} = 1 + LG,$$

$$P = \frac{K}{K + H_0} \cdot \frac{LG}{1 + LG} \cdot 100,$$

where ΔpH = pH depression at equilibrium,

L = equilibrium constant,

G = molar concentration of *N*-acetyl-D-glucosamine,

P = percentage combination,

K = apparent dissociation constant of the amino acid or peptide, and

H_0 = initial hydrogen ion concentration of reaction mixture.

Figure 4 records the course of pH depressions with time in the interaction of *N*-acetyl-D-glucosamine with glycine, L-tyrosine, L-lysine, and glycylglycine for an initial pH value of 9.0 and with L-leucylglycine for an initial pH value of 8.68. Figure 5 gives the increase in percentage combination with time. The equilibrium constants (L) for these reactions at the pH values given above are: glycine 9.7, L-tyrosine 9.0, L-lysine 3.4, glycylglycine 1.4, and L-leucylglycine 3.1. The apparent dissociation constants given by Cohn and Edsall (1943) were used to calculate the percentage combination. Equilibrium was reached in 24 hr except for L-lysine, where there was a continued very small increase. This could be caused by reaction at the ϵ -amino group. Micheel and Klemer (1951) have reported that only the α -amino group of lysine reacts with 1-fluoro-D-glucose; the ϵ -amino group does not react. It can be seen that the percentage combination for the peptides is greater than that for the amino acids. Also the percentage combination for tyrosine and lysine are similar and both are more than twice that of glycine. This is due to the differences in the magnitudes of the dissociation constants of the amino acids and peptides, as well as to differences in the equilibrium constants of the reactions because the dissociation constant determines the initial amount of the reagent. The results show clearly that the differences in the degrees of ionization are more important in determining the percentage combination than are the differences in the equilibrium constants. In the past it has generally been considered that, in

contrast with many other amino acids, tyrosine manifests a strong affinity towards and forms compounds with polysaccharides in the biological pH range (Geidroye, Cichocka, and Mystkowski 1935). This conclusion thus receives further support.

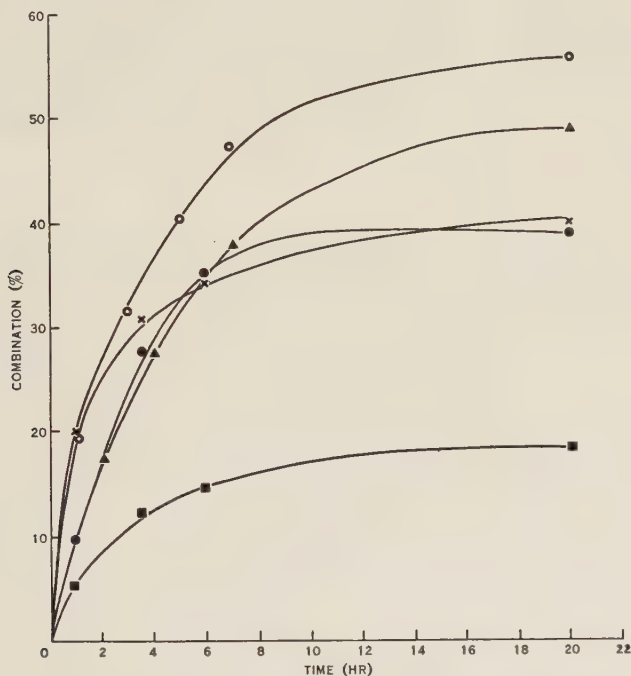
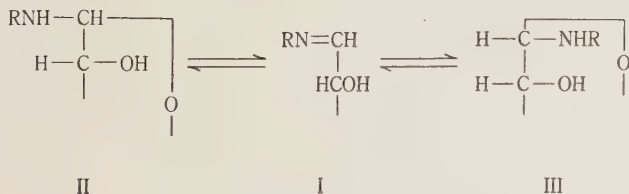


Fig. 5.—Percentage combination of *N*-acetyl-D-glucosamine (1M) with amino acids (0.1M) and with peptides (0.05M) as determined by measurement of pH depression.

- — ■, Glycine, initial pH 9.0 and 18°C.
- — ●, L-Tyrosine, initial pH 9.0 and 18°C.
- × — ×, L-Lysine, initial pH 9.0 and 18°C.
- — ○, L-Leucylglycine, initial pH 8.68 and 22°C.
- ▲ — ▲, Glycylglycine, initial pH 9.0 and 22°C.

The non-enzymic condensations of amino acids with aldoses such as glucose have been extensively studied. It has been established that the initial reaction



is one in which the aldose reacts with the amino acid in equimolecular ratio, the reaction product being a Schiff base (i.e. an azomethine) (I) although it may be in the isomeric *N*-glycoside form (II or III). From the experimental

evidence presented in this paper it is concluded that the reaction between amino acids and *N*-acetyl-D-glucosamine is of the same type, i.e. a Schiff base is formed from equimolar quantities of each reactant. The reaction mechanism involves the addition of a nucleophilic reagent (the amino acid) to a carbonyl group (the *N*-acetyl-D-glucosamine) with the subsequent elimination of water from the addition product although rearrangement may take place to give an *N*-glycoside. In aqueous solutions such a reaction would be reversible. Under acidic conditions amino acids and peptides are almost completely ionized so it follows that almost no combination would occur, which is in agreement with all known facts.

TABLE 1

THE BINDING OF *N*-ACETYL-D-GLUCOSAMINE BY INSECT CUTICULAR PROTEINS AT 37°C AND pH 8.77 AS DETERMINED BY THE METHOD OF EQUILIBRIUM DIALYSIS

	Protein Tube		Control Tube	
	Outside Bag	Inside Bag	Outside Bag	Inside Bag
Volume of solution	20 ml	10 ml	20 ml	10 ml
Protein (mg)		19.0		
Concn. <i>N</i> -acetyl-D-glucosamine	1.953 mM		2.052 mM	2.052 mM
Log free concn. <i>N</i> -acetyl-D-glucosamine	-2.7093			
<i>N</i> -acetyl-D-glucosamine bound (mg)		0.66		
<i>N</i> -acetyl-D-glucosamine bound per g protein (mg)		34.7		
pH of solution	8.77	8.77	8.77	8.77

N-acetyl-D-glucosamine is definitely bound by the water-soluble protein isolated from larval cuticles of *D. dorsalis*. The affinity of this protein for *N*-acetyl-D-glucosamine at pH 8.77 and 37°C is shown in Figure 6 where *N*-acetyl-D-glucosamine bound per g protein has been plotted against the logarithm of the free concentration of *N*-acetyl-D-glucosamine. A typical set of results from which these graphs have been drawn is given in Table 1. With a concentration of free *N*-acetyl-D-glucosamine of 1.95mM the protein binds 3.3 per cent. of its own weight of *N*-acetyl-D-glucosamine. No binding of *N*-acetyl-D-glucosamine could be detected at pH 5.5 and 37°C, and the addition of zinc nitrate (cf. Klotz and Loh Ming 1954) at pH 5.5 did not cause any binding to take place. In order to obtain information on the types of groupings which may be involved in the binding which occurs at alkaline pH, the protein was both iodinated and acetylated. Acetylation would inactivate the free α -amino groups while iodination would affect the tyrosine residues. From Figure 6 it can be clearly seen that the iodinated protein shows a greater loss of affinity for *N*-acetyl-D-glucosamine than does the acetylated protein. Clearly the tyrosine groups as well as the free amino groups are involved in the interactions of the protein with *N*-acetyl-D-glucosamine. It has been shown that this cuticular

protein has a high tyrosine content and that the free α -amino groups are located on glutamic acid, glycine, serine, and alanine residues while the ϵ -amino group of lysine is also free (Hackman 1953a).

It can be concluded that α -amino acids, peptides, and proteins react with *N*-acetyl-D-glucosamine. No reaction occurs under acidic conditions but under alkaline conditions the amount of reaction increases as the pH increases. At a given pH value the amount of combination depends upon the equilibrium con-

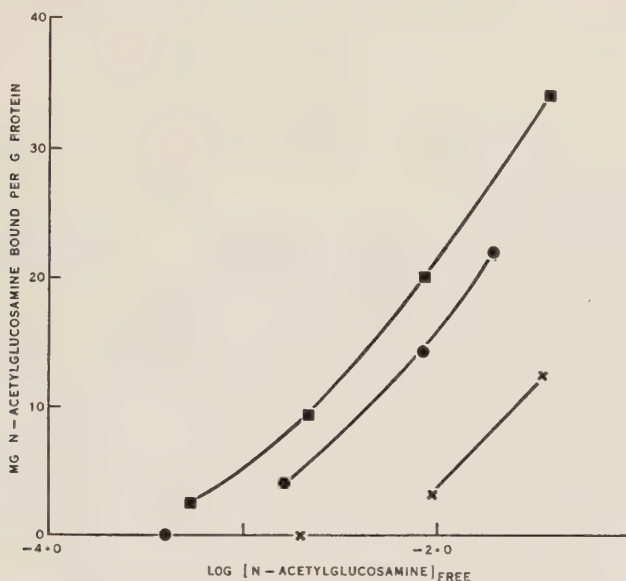


Fig. 6.—Binding of *N*-acetyl-D-glucosamine by proteins at 37°C and pH 8.77. Protein concentration 0.19 per cent.

- — ■, Insect cuticular protein.
- — ●, Acetylated insect cuticular protein.
- × — ×, Iodinated insect cuticular protein.

stant of the reaction and upon the dissociation constant of the α -amino nitrogen as this determines the initial concentration of the reagent. Differences in degrees of ionization are more important than differences in the equilibrium constants. Tyrosine, as well as free α -amino groups, appears to play an important part in the binding of *N*-acetyl-D-glucosamine by the cuticular protein. The compounds formed are of the Schiff base (or azomethine) type although they may be in the form of *N*-glycosides. They are unstable, being split even in the biological pH range. The reaction is therefore of a similar type to that which occurs between glucose and α -amino acids or peptides.

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KERATIN DERIVATIVES EXTRACTED FROM WOOL WITH ALKALINE THIOLYCOLLATE SOLUTIONS

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Summary

In extension of previous work (Gillespie and Lennox 1953), the conditions under which proteins may be extracted from washed Merino wool have been further examined. Approximately 65 per cent. of the wool can be dissolved by a 40-min extraction at 50°C with 0.1M thioglycollate at an initial pH of 12.6. Electrophoresis at pH 11 in thioglycollate-glycine buffer indicated the presence of seven minor and one major component, the latter amounting to 41 per cent. of the wool. The minor components can be completely removed from the wool by five 20-min extractions with 0.1M thioglycollate at an initial pH of 10.5. Extraction of the residue at pH 12.3 yields the major component. This moves as a single peak on electrophoresis between pH 8.0 and 12.0 in the presence of various buffers. It has a mobility of $-7.2 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$ at a protein concentration of 0.5 per cent. in thioglycollate-glycine buffer of ionic strength 0.22 at pH 11.0. At higher protein concentrations there is anomalous behaviour on the descending boundary and this can be prevented by increasing the ionic strength or replacing thioglycollic acid with mercapto-ethanol. The ascending pattern is unaltered by these changes or by increased protein concentration.

I. INTRODUCTION

A study of the protein constituents of wool is being made in an attempt to assess the contribution to the properties of wool made by individual proteins.

The insolubility of wool in aqueous media constitutes a major impediment in studying its properties. Of the known reagents for converting the proteins of keratinous tissues, such as wool, into soluble derivatives, an alkaline solution of thioglycollic acid is perhaps the most satisfactory. For example, Goddard and Michaelis (1934) found that solutions of thioglycollate extracted greater amounts of soluble protein from wool than did solutions of sulphide or cyanide, and they expressed the view that "it simply reduces disulphide to sulphydryl groups with no other appreciable change." The superior keratin-dispersing action of thioglycollate is likely to be associated with its greater disulphide-bond-splitting activity than equivalent concentrations of cysteine or cyanide (Lennox and Forss 1953).

The partial solution of wool in alkaline thioglycollate solutions has provided a means of investigating the wool dispersions by methods commonly used for characterizing soluble proteins.

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It has previously been reported that the extracts obtained by treating wool with thioglycollate at an initial pH of 12.6 contain at least three components, two of which can be removed by fractional extraction at pH 10.5 before extraction at the higher pH (Gillespie and Lennox 1953). Further details of this work and an extension of the thioglycollate extraction and electrophoretic studies are given in the present paper.

II. MATERIALS AND METHODS

Merino wool 64's quality from which the tip had been removed was extracted with ethanol, washed repeatedly in distilled water, and dried in warm air. In large-scale preparative experiments wool from the same bale was used in the form of dry combed top. This was extracted six to eight times with light petroleum, twice with ethanol, and repeatedly with distilled water before being dried in warm air.

Except where otherwise stated 30 ml of solution was used to extract each gram of wool and all extractions were made for 20 min at $50^{\circ}\text{C} \pm 0.1^{\circ}$. Potassium salts were used throughout to minimize salt errors during pH measurements in the alkaline region. A standard glass electrode assembly was used. The thioglycollic acid was of laboratory reagent grade and solutions were standardized before use by standard iodometric procedures. The wool extracts were stored by freeze-drying and sealing in air-tight containers.

In large-scale experiments the protein solutions were adjusted to the pH of maximum precipitation and the precipitate allowed to settle, the supernatant decanted off, the precipitate quickly dissolved in one-tenth of the original volume of 0.1M thioglycollate at pH 10.5, and this concentrated solution freeze-dried and sealed into air-tight containers.

The amount of wool passing into solution was measured by estimating the total nitrogen in the liquor by the micro-Kjeldahl method and by estimating the loss in weight of the wool. The partly digested wool was repeatedly washed with water to remove soluble degradation products, prior to drying it at 102°C and weighing. Using a factor of 6.25 to convert nitrogen percentage to protein, the results obtained by the two methods agree closely (Fig. 1) for digestion to about the 60 per cent. level but diverge to some extent at higher degrees of digestion.

The pH-solubility relationships of the wool proteins were determined by precipitating the proteins in each extract at a series of pH values in the range 4-11. The final protein concentration and ionic strength were kept constant and the temperature maintained at 2°C . After 18 hr equilibration the precipitates were centrifuged down and dissolved in 0.1M carbonate solution. The wool protein solutions were not optically clear at pH values normally used for measuring concentration—utilizing the absorption near $280\text{ m}\mu$ by the aromatic amino acids. An aliquot of this solution was therefore partly hydrolysed by boiling with an equal volume of 5N HCl for 30 min and the absorption at $275\text{ m}\mu$ measured. To correct for the absorption at this wavelength by the thioglycollic acid present, the protein in a second aliquot was precipitated by half saturation

with ammonium sulphate, the supernatant from the precipitate hydrolysed with HCl, and the absorption also measured at $275\text{ m}\mu$. The difference between the two values was converted to the equivalent protein concentration using the extinction coefficient of $E_{1\text{ cm}}^{1\%} = 8.9$ (air-dry weight).

Electrophoresis of wool proteins was carried out in a Tiselius apparatus at 1°C , using a constant potential gradient in the \square tube of about 2.5 V/cm . The diagonal schlieren method was employed, using as a light source a mercury vapour lamp from which monochromatic light ($\lambda = 546\text{ m}\mu$) was isolated by means of a filter. The curves were photographed on Ilford 35-mm panchromatic film (FP3). The wool extracts (25 ml) were dialysed in "Visking" sausage casing (24/32 in. size) against 2 l of buffer solution for at least 2 days at 2°C . Protein

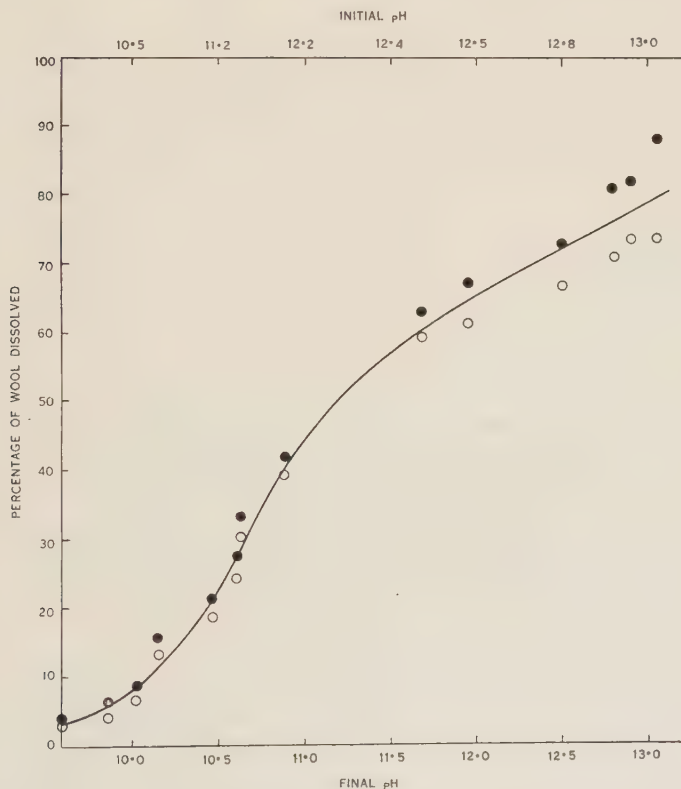


Fig. 1.—Effect of pH on the solution of wool in 0.1M thioglycollate at 50°C for 40 min. O, Estimated from N dissolved.

●, Estimated from weight of undigested wool.

concentrations were determined refractometrically. It was found that a reducing agent was needed in the buffer to prevent oxidation of these sulphydryl proteins. Oxidation was manifested by gelling within 24 hr. Thioglycollic acid or mercaptoethanol at concentrations as low as 0.005M could prevent this oxidation but for routine experiments somewhat higher concentrations were

used. The standard buffer had an ionic strength of 0.22 and consisted of 0.05M thioglycollic acid, 0.1M glycine with KOH added to give pH 11.0.

Mobilities were calculated from measurements made on the descending boundaries at protein concentrations of about 0.5 per cent. The relative concentrations of various components were calculated from the ascending boundaries because of the superior resolution at this boundary and the freedom from anomalous effects.

III. EXPERIMENTAL

It should be noted that all the pH values referred to in this paper are initial pH's; the final values and presumably those prevailing during the major part of the extraction are somewhat lower (see Fig. 1).

TABLE 1
COMPARISON OF DISPERSING ACTION ON WOOL OF 0.1M THIOGLYCOLLATE WITH 0.5M THIOGLYCOLLATE, AT TWO pH VALUES

Thioglycollate concentration (M)	0.1	0.1	0.5	0.5
Initial pH value	11.6	12.5	11.5	12.5
Final pH value	10.6	12.4	11.2	12.2
Total N in extract (mg N per ml)	1.23	2.59	0.73	1.26
Equiv. protein extracted (%)	23	49	14	24

(a) Preparation of Thioglycollate Extracts

(i) *Influence of pH.*—The quantity of wool dispersed in 0.1M thioglycollate in 20 min at 50°C increased sharply as the initial pH was increased from 10.5 to 12.3 but thereafter less rapidly (Fig. 1). By dialysing 5-ml portions of the extracts in Visking sausage casing against 5 ml of distilled water for 7 days at 2°C and measuring nitrogen content and volume of both solutions, it was shown that the extracts prepared at pH values below 12.0 contained only traces of dialysable nitrogen, whilst those made at pH 12.6 lost about 3 per cent. of total N and those at pH 13.0 about 50 per cent.

Pretreatment of the wool in alkali at pH 10.6 for 24 hr at 50°C caused a decrease in the amount of protein extracted. The pretreatment was shown to give rise to lanthionine in the wool. However, no lanthionine was present in either the solutions or in the residues produced during thioglycollate extractions.

(ii) *Comparison of Different Cations.*—Thioglycollic acid solutions were adjusted to a final thioglycollate concentration of 0.1M at pH 11.0, using LiOH, NaOH, KOH, NH₄OH, and Ca(OH)₂. The solutions containing univalent cations dispersed 15-20 per cent. of the wool under standard conditions whereas the Ca(OH)₂ solution dispersed only 8 per cent.

(iii) *Effect of Thioglycollate Concentration.*—Solutions containing potassium thioglycollate varying in concentration from 0.1 to 1.0M, at an initial pH of 11.0, were used to extract protein from wool under the standard condi-

tions of time and temperature. It was found that extent of extraction increased with increasing thioglycollate concentration to a maximum at about 0.7M (Fig. 2). During extraction the pH decreased to values ranging from 9.7 for 0.1M to 10.8 for the 0.5M solution.

The increase in the solubility of the wool protein with increase in pH and decrease with increase in the thioglycollate concentration above a certain maximum value is confirmed by an experiment reported in Table 1. It will be noted that extraction at higher pH values than those reported in Figure 2 lowered the optimum concentration of thioglycollate for extraction to less than 0.5M.

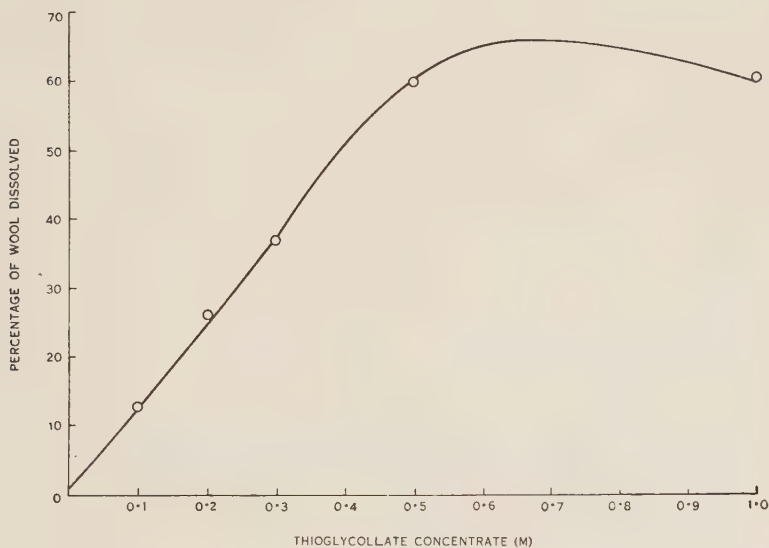


Fig. 2.—Effect of thioglycollate concentration on the solution of wool. Initial pH 11. Heated for 40 min at 50°C.

(iv) *Effect of Time of Extraction.*—Figure 3 shows the relationship between time and extent of digestion at different pH values. At the higher pH values tested, i.e. 12.0 and 12.3, the reaction proceeded in two stages, the first of which was completed in less than an hour with 60-70 per cent. of the wool extracted and the second still incomplete in 40 hr with 90 per cent. of the wool digested.

(v) *Effect of Temperature.*—Increase in temperature of the 0.1M thioglycollate solution at an initial pH of 11.7 had little effect on its dispersing action up to 40°C (Fig. 4) but thereafter increased it sharply up to about 60°C.

Measurement of digestion at intervals during extraction in pH 10.7 thioglycollate at 33.0, 49.2, and 64.0°C (Fig. 5) enabled the curve in Figure 6 to be determined from which the activation energy for the extraction was calculated to be 7.4 kcal per mole.

(vi) *Extraction of Disintegrated Wool and Cortical Cells.*—Wool ground to 40 mesh in a Wiley mill was somewhat more easily extracted by 0.1M thioglycollate at pH 12.5 than was whole wool. At 50°C, 68 per cent. of the ground

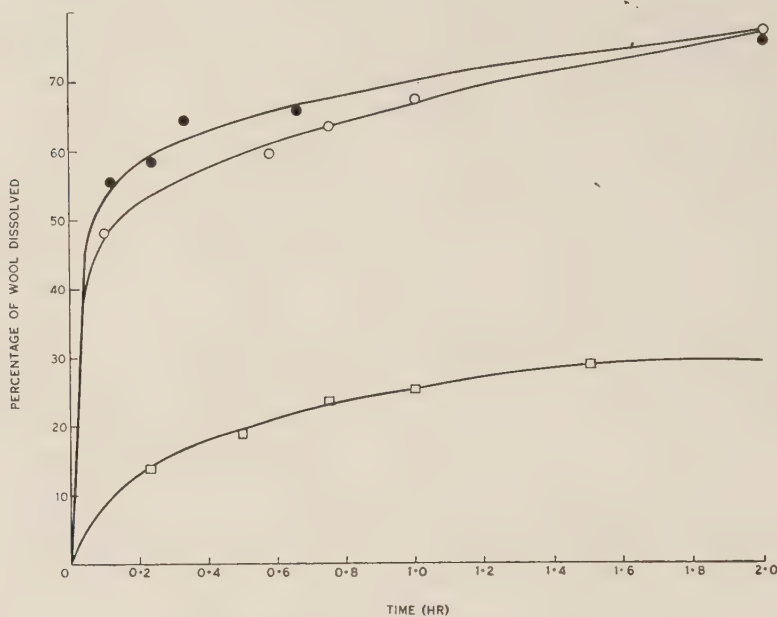


Fig. 3.—Effect of time on solution of wool in 0.1M thioglycollate at 50°C.
□, Initial pH 10.5. O, Initial pH 12.0. ●, Initial pH 12.3.

TABLE 2

PERCENTAGE PROTEIN EXTRACTED FROM DISINTEGRATED AND INTACT WOOL DURING 20 MIN AT 50°C IN 0.1 M THIOGLYCOLLATE SOLUTIONS

Experiment No.	Cortical Cells from Trypsin-Digested Wool		Finely Ground Wool*		Intact Wool	
	pH 10.4 15	pH 11.2 24	pH 10.4 22	pH 11.2 44	pH 10.4 8	pH 11.2 45
2	pH 10.5 19	pH 12.3 27	pH 10.5 24	pH 12.3 53	pH 10.5 13	pH 12.3 62

* The protein digestion was corrected to allow for the presence of 9 per cent. powdered glass in this preparation.

wool dissolved as compared with 62 per cent. of whole wool in a 40-min. extraction. Table 2 shows the results obtained in a similar experiment, in which an extraction was made on cortical cells from trypsin-damaged wool and ground

wool which had been broken down by shaking with glass beads in water for 16 hr.

(vii) *Mechanical Agitation During Extraction.*—When wool was treated with 0.1M thioglycollate at pH 12.6 for 20 min at 50°C, and the reaction mixture disintegrated for 2 min in a Waring Blender and then heated for a further 20 min at 50°C, 70 per cent. of the wool protein was dispersed. These extracts were very cloudy and they could not be clarified by filtration or centrifugation at 25,000g. Electrophoresis revealed a normal pH 12.6 pattern (Gillespie and Lennox 1953).

Agitation of the wool by continuous tumbling in tubes immersed in the thermostat bath for 40 min at 50°C increased the protein extraction in 0.1M thioglycollate at pH 11.6 from 13 per cent. in stationary tubes to 15 per cent. and with 0.1M thioglycollate at pH 12.8 from 57 to 62 per cent.

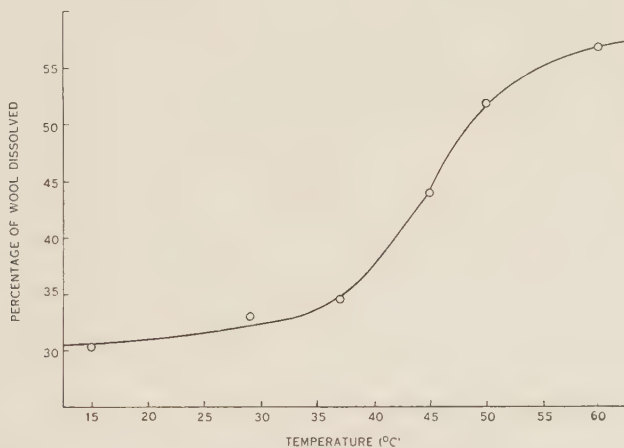


Fig. 4.—Effect of temperature on solution of wool in 0.1M thioglycollate. Initial pH 11.7. Heated for 40 min.

(b) Fractional Extraction of Wool with Thioglycollate

An extract of wool made with 0.1M thioglycollate at pH 12.6 for 20 min at 50°C contains most of the easily extractable protein (see Fig. 3). Electrophoresis shows that it contains at least four components, the second of which is the major one. Preliminary experiments showed that comparable amounts of protein could be obtained in solution by stepwise extraction at a number of lower pH levels. Furthermore, it was found that the minor components (1, 3, and 4) could be more readily extracted at low pH values than the major one (2), and that by using a series of extractions at an initial pH of 10.5, all these minor components could be removed from the fibre with only partial loss of component 2.

Subsequently the procedure followed was to extract the wool five times for 20 min at 50°C with thioglycollate solutions at an initial pH of 10.5, followed by one extraction with a solution of 12.3. The first extract contained

only 8.5 per cent. of the wool, the final pH being comparatively low, the second contained about 15 per cent. of the wool, the amount dissolved decreasing in subsequent extracts to 3 per cent. of the wool in the fifth extract. The sixth extract made at an initial pH of 12.3 contained almost 20 per cent. of the wool protein and appeared to consist of only one protein. These results are summarized in Figure 7.

In another experiment (Fig. 8) the first five extractions at an initial pH of 10.5 (A-E) were followed by five extractions (F-J) with 0.1M thioglycollate at pH 12.3, three further extractions with thioglycollate at pH 13.0 (K-M), and one with 1.0M KOH (N), each extraction being for 20 min at 50°C. The insoluble residue was then dissolved by boiling for 2 min in one-third of the standard volume of 1.0M KOH (O).

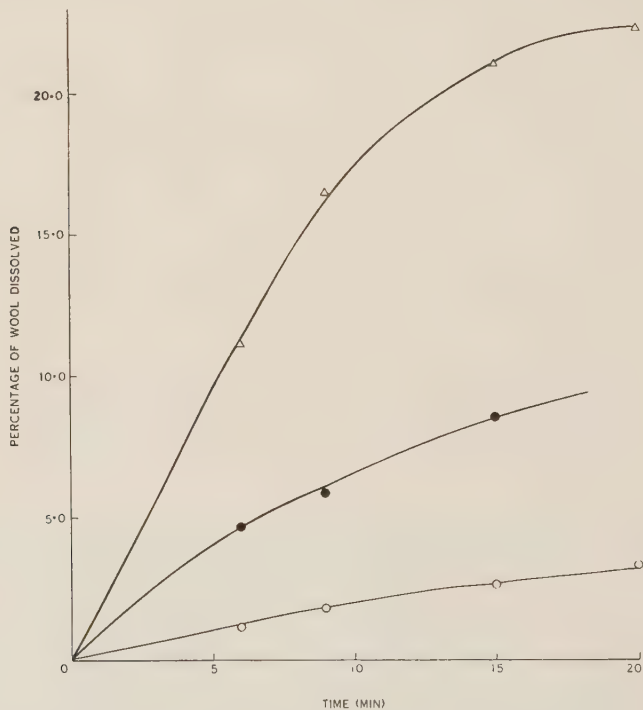


Fig. 5.—Effect of time on solution of wool in 0.1M thioglycollate. Initial pH 10.5. \circ , At 33°C. \bullet , At 49.2°C. Δ , At 64°C.

Dialysis of 5-ml portions of the extracts in "Visking" sausage casing in running tap water with agitation removed less than 10 per cent. of the nitrogen from extracts A-M. With extract N, however, 24 per cent. dialysed away and with extract O, 28 per cent. When 5-ml portions of the extracts were dialysed without agitation for 14 days at 2°C against 5 ml distilled water and the nitrogen content of the solutions was measured on either side of the membrane, making corrections for volume changes, less than 0.1 per cent. of the total N was

dialysable from the pH 10.5 extracts (A-E) and 2-3 per cent. from the pH 12.3 extracts (F-J).

(c) *Precipitation Curves of Extracted Wool Proteins*

The thioglycollate wool proteins are readily precipitated by acidification of their solutions, the pH necessary for maximum precipitation varying with the type of material. The pH-solubility relationships of the wool proteins were determined in solutions of equal protein concentration (1 per cent.) containing 0.05M thioglycollate, the pH value having been adjusted with acetic acid. After equilibration for 18 hr at 2°C, the solutions were centrifuged at this temperature and the protein in solution, and in the precipitate, estimated by the spectrophotometric method. The results are shown in Figure 9.

A small proportion of the protein in the first 10.5 extract (approx. 2 per cent.) was not acid-precipitable; however, all subsequent extracts appeared to be completely precipitable. It can be seen (Fig. 9) that the proteins in the pH 12.3 extracts, and those made at pH 13.0, were soluble to a slight extent on the acid side of the point of maximum precipitation. However, the solubility was too low for a satisfactory electrophoresis run to be made at these pH values.

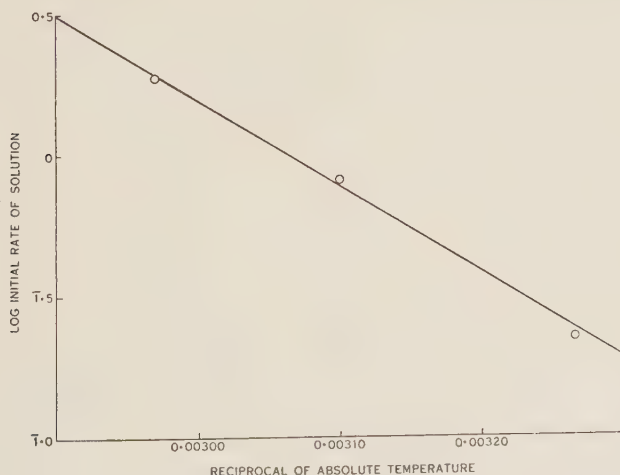


Fig. 6.—Relationship between logarithm of initial rate of extraction and the reciprocal of absolute temperature. Data from Figure 5.

(d) *Properties of the Wool Proteins*

The freeze-dried proteins obtained from the thioglycollate extraction of wool are sulphhydryl proteins, colourless, freely soluble in water at pH values above the precipitation range, but readily oxidized to give insoluble materials. By dialysing a solution containing 1 per cent. protein or more against a large volume of water, in order to remove the potassium thioglycollate always present in these preparations, a firm gel was formed and at this stage there were no detectable SH groups present. This gelling was not prevented by "Versene." The

solutions turn yellow on standing at room temperature, presumably owing to the release of sulphur from the cystine residues at the alkaline pH. This reaction is known to proceed more readily with cystine in peptide combination than with the free amino acid (Brand and Sandberg 1926). The exact explanation for the yellowing is not known; however, a strong absorption band appears at $330\text{ m}\mu$ which increases in intensity as the reaction proceeds. The alkaline thioglycollate extractant on storage also produced a yellow colour but only in small amount.

The freshly acid-precipitated solids were readily redissolved in thioglycollate at pH 10.5 and these solutions were then freeze-dried for storage. If, however, the solid was packed down by centrifuging, by pressing, or by vigorous stirring, the material became almost insoluble in the thioglycollate and what did dissolve became insoluble after freeze-drying. Compression of the gel probably favoured the formation of relatively stable cross-linkages between the protein micelles. I. J. O'Donnell (personal communication) has observed similar behaviour with some peracetic acid-oxidized wool protein. This material in the acid-precipitated state had to be treated gently and not pressed, otherwise it did not readily dissolve in 0.1N ammonia but merely swelled with some slow dissolution.

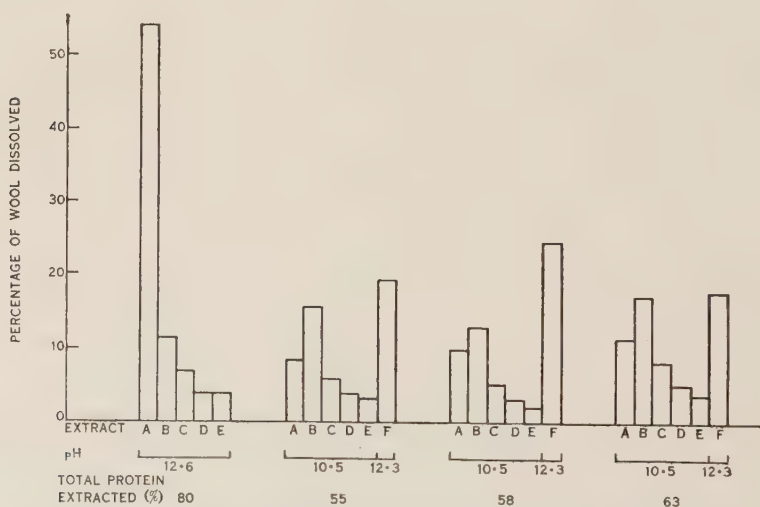


Fig. 7.—Histograms showing the percentage of wool solubilized by successive extractions with 0.1M thioglycollate, each for 20 min. at 50°C .

(e) *Electrophoresis of the Wool Proteins*

Electrophoresis patterns obtained with successive thioglycollate extracts, run in the standard buffer at pH 11, are shown in Figure 10. It can be seen that the complexity of the patterns progressively decreased and the sixth extract (F) was a relatively pure material. Four conspicuous components appeared in the first 10.5 extract (A). Of these the slowest, component 1, was not homo-

geneous and was resolved in this and many other preparations into at least three components. When this extract was precipitated at pH 6, a portion of component 1 remained in the supernatant. Electrophoresis of this supernatant

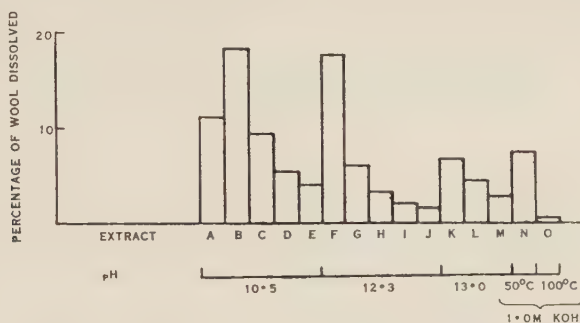


Fig. 8.—Histogram showing the distribution of wool protein in 15 successive extractions, 13 (A to M) with 0.1M thioglycollate.

fraction showed only a broad low peak after some hours' running and this peak did not appreciably sharpen when the current was passed in the reverse direction for an equal time. Furthermore, the amount of protein detectable on electrophoresis, corresponding to component 1, decreased as the time of dialysis increased. From this evidence and that of Harrap (1955) it seems that the first extract is very heterogeneous and contains some proteins of relatively low molecular weight.

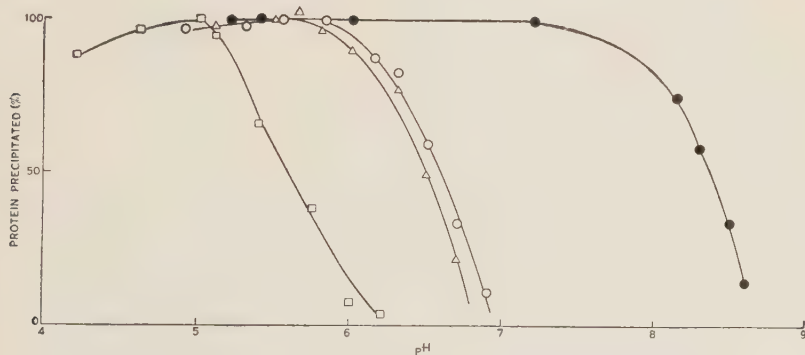


Fig. 9.—pH-solubility curves of wool proteins. ●, First pH 10.5 extract (A). ○, Second pH 10.5 extract (B). △, Subsequent pH 10.5 extracts (C, D, E) and pH 12.3 extracts (F, G, H, I). □, First and second pH 13 extracts (K, L).

The second, third, fourth, and fifth pH 10.5 extracts contained one major component (2) and decreasing amounts of the others (1, 3, 4). The first pH 12.3 extract made on the residue, then yielded a material which on electrophoresis moved as a single peak. This peak was very symmetrical and it showed little spreading and no splitting during a 6-hr electrophoresis run at pH 11 in the standard buffer.

(f) Variation in Extraction Conditions

Attempts to increase the yield of component 2 by prolonging the first extraction in pH 12.3 thioglycollate (extract *F*) to 18 hr at 50°C or by using thioglycollate at pH values greater than 12.3, or buffered at pH 12.3, for the extraction were unsuccessful. In each case the electrophoresis pattern showed a "spike" component on the leading edge of component 2. A similar pattern was obtained by heating extract *F* at 50°C after removal of the wool residues (Fig. 11).

(g) Location of the Component 2 in the First pH 10.5 Extract

The first pH 10.5 extract contains a complicated mixture of proteins and some trouble was experienced in determining the exact location of the pure component in the electrophoretic pattern of this extract. From the values calculated for the mobilities of the pure component and the four main proteins in the mixture it was previously reported that the pure component was located

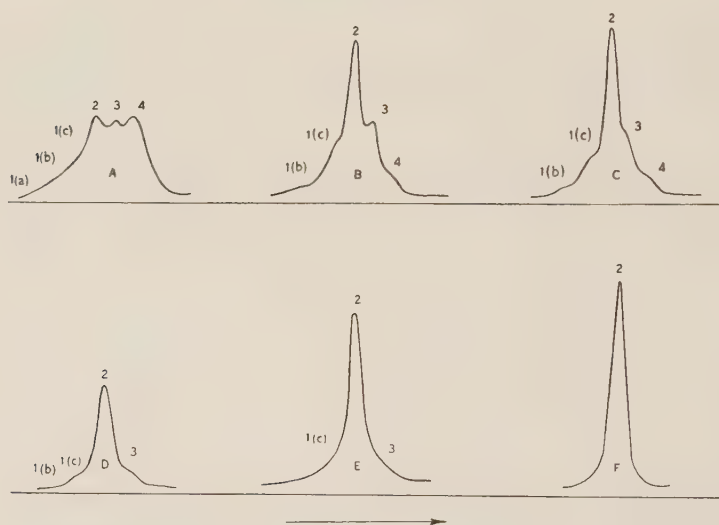


Fig. 10.—Electrophoretic patterns of wool proteins. Ascending boundaries shown. Run at pH 11 in 0.22 $\Gamma/2$ buffer. A-E are successive pH 10.5 extracts, *F* is a 12.3 extract. The component numbers are shown over the appropriate peaks.

in the first peak (Gillespie and Lennox 1953). However, on adding a preparation of component 2 to the first pH 10.5 extract, the second peak of the electrophoresis pattern was greatly increased in height (Fig. 12), showing this to be identical with the pure component. It is well known that on electrophoresis of a mixture of proteins, only the mobility of the leading component can be calculated with accuracy (Svensson 1946; Armstrong, Budka, and Morrison 1947). This, together with the dependence of mobility on protein concentration, accounts for the difficulties encountered in the present investigations.

(h) Percentage of Electrophoretic Components in Whole Wool

Having identified the major component, the other components were also identifiable by their relative positions in the electrophoretic pattern of each extract. By measuring the area under each peak, the percentage of the total wool protein in each component was calculated. These results are shown in Table 3. In addition to the three sub-components of component 1, a trace of a faster-moving component, designated 5, was often found.

TABLE 3
PERCENTAGE OF WOOL PROTEIN CORRESPONDING TO EACH COMPONENT
Data from electrophoretic patterns in Figure 10

Component	Proportion of Each Component Present in Each of the Following Extracts (%)						Wool Equivalent to Each Electrophoretic Component (%)
	A	B	C	D	E	F	
1 (a)	6	2	—	—	—	—	1.1
1 (b)	7	8	8	2	—	—	3.1
1 (c)	8	12	14	9	5	—	5.1
2	30	42	56	72	90	100	41.4
3	20	21	14	13	5	—	8.3
4	26	15	8	4	—	—	6.6
5	3	—	—	—	—	—	0.3
Wool equivalent to each extract (%)	11.2	18.3	9.5	5.2	4.0	17.7	Total 65.9 (%)

(i) Studies on the Electrophoresis of the Pure Component

Electrophoresis of the pure component, i.e. component 2, in the standard buffer at pH 11 showed, on the ascending boundary, only a single symmetrical peak. The descending boundary showed some anomalous effects and experiments were made to explain these.

The electrophoretic mobility of the protein was found to be -7.2×10^{-5} cm² V⁻¹ sec⁻¹ at 0.5 per cent. protein concentration in the standard buffer at pH 11, but on increasing the protein concentration to 1 per cent. the mobility decreased to -6.3 , and even lower mobilities were recorded at higher protein concentrations. The descending boundary gave an anomalous multiple peak pattern at protein concentrations greater than 1 per cent., whereas a normal symmetrical peak was obtained at concentrations of about 0.5 per cent. or less.

On electrophoresis of component 2 in thioglycollate solutions containing 1.5 per cent. protein and NaCl to adjust the ionic strength to 0.02, 0.05, 0.10, 0.20, and 0.50, the single component pattern previously observed in the ascending limb was unchanged. The anomalous pattern in the descending limb, how-

ever, was progressively clarified with increase in ionic strength and at $\Gamma/2 = 0.5$ only a single peak appeared, similar to that seen in the ascending limb pattern.

Replacement of thioglycollate in the electrophoresis buffer with 0.05M mercaptoethanol or with 0.025M sodium sulphite did not affect the pattern in the ascending limb but, like high ionic strength, eliminated the anomalous components in the descending limb.

Electrophoresis of solutions of component 2 in which the thioglycollate concentration varied from 0.005M to 0.10M, with a constant ionic strength of 0.22, yielded normal single-component patterns in the ascending limb and the usual anomalous pattern in the descending limb.

The patterns were unchanged when glycine was replaced by piperidine-HCl, K_2CO_3 , or K_2HPO_4 - K_3PO_4 in an electrophoresis buffer of the usual ionic strength and pH.

Electrophoresis at pH values between 8.0 and 12.0 yielded normal patterns except that as the pH fell below 9 the peaks sharpened, perhaps owing to the tendency of the solutions to gel at low pH values. $KHCO_3$ was used in the thioglycollate solutions at pH 8.0, veronal at pH 8.8, and glycine at all higher pH values.



Fig. 11.—Electrophoretic pattern showing modification of component 2 after heating extract *F* at 50°C for 18 hr.

IV. DISCUSSION

The most interesting result of these studies has been the preparation of a protein from wool (component 2) which constitutes over 40 per cent. of the total fibre. It will almost certainly be directly or indirectly responsible for many of the properties of the wool fibre.

This fraction has been run in electrophoretic experiments under as wide a variety of conditions as possible, involving changes in pH and buffer composition. Under all these conditions the material appears to be homogeneous. It has not been possible to run the protein at pH values below its isoelectric point because of its insolubility, nor to run it in the absence of a reducing agent,

consequently it has not been possible to carry out more critical tests for homogeneity.

Electrophoresis of these wool proteins has provided many problems, chief of which has been the anomalous production of new peaks on the descending boundary with increase in protein concentration. The fact that this phenomenon can be repressed or eliminated by increasing the salt concentration or by lowering the protein concentration is consistent with its being an association process.

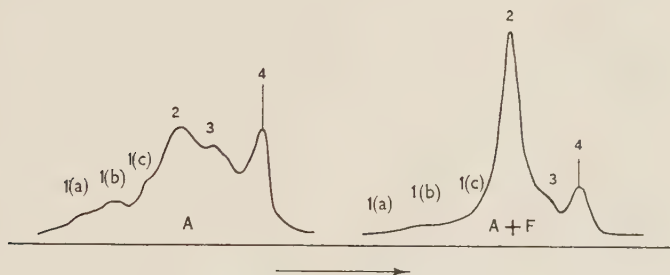


Fig. 12.—Electrophoretic patterns showing the identification of the pure component 2 in the first pH 10.5 extract by mixing extract F with A to enlarge the component 2 peak.

In some extracts, particularly those prepared with pH values at 13 and higher, the electrophoresis patterns show characteristic spiked peaks in both ascending and descending patterns. The reason for their appearance is unknown although they have been observed previously with other proteins, thus Taylor, Green, and Cori (1948) found a spike in the descending boundary pattern of crystalline aldolase, Hamoir (1953) in the ascending pattern of muscle extracts, attributed to turbidity and high viscosity of the solutions, and Morrison (1954) in both boundaries of heart muscle aconitase. A spike can also be observed in the β anomaly of plasma in the descending boundary (Abramson, Moyer, and Gorin 1942). These phenomena may be unrelated but in certain cases they appear to be caused by association-dissociation processes.

A study of the kinetics of the extraction process indicated an initial rapid solubilization of some 70 per cent. of the wool protein with a subsequent slower extraction of more resistant material. This agrees with the observations of Jones and Mecham (1943) and Booth (1952). The initial rapid reaction corresponds with the appearance of components 1-4 and appears to cease when these have been completely extracted. At this stage microscopical examination showed a grossly swollen tube-like structure with the scale markings still visible and many of the internal cell membranes intact (Fraser and Rogers 1953). This suggests that the high resistance of the residue to thioglycollate extraction as compared with the bulk of the fibre is due to a fundamental difference in its chemical structure.

The first two pH 10.5 thioglycollate extractions caused the S segment to swell and lose birefringence but had little effect on the birefringence of the H segment. This birefringence disappeared during the subsequent pH 10.5 extractions and the fibre swelling increased. It would seem that a number of processes are involved, firstly the diffusion of thioglycollate and OH ions through the membranes into the fibre, then the solubilization of the proteins accompanied by swelling, followed by diffusion of the soluble proteins out. The work of Harrap (1955) suggests that diffusion is a rate-limiting process in the extraction and this is confirmed by the value of 7.4 kcal obtained for the activation energy of extraction using pH 10.7 thioglycollate. This lies within the range of values 4.5-8.0 which typifies the diffusion of ions through a liquid layer. Values within this range have been reported for the diffusion into wool of potassium permanganate (Alexander and Hudson 1949), chlorine (Alexander, Gough, and Hudson 1949), and dyes (Alexander and Hudson 1950). Thus the recovery of proteins from wool with thioglycollate is likely to be diffusion-controlled, the limiting step being either the ingress of the reagent or the egress of protein through a diffusion layer.

A further illustration of the importance of diffusion in the extraction of wool proteins was the repression of the extraction process at high thioglycollate concentrations (Table 1), corresponding with similar observations by Booth (1952). It is also related to the lower depilation and wool-dispersing action of 2.0M Na₂S as compared with 1.0M Na₂S (Gillespie 1951), and to the repressive effect of Na₂SO₃ (Gillespie 1953) on the depilatory action of enzymes from *Aspergillus oryzae* at concentrations exceeding 1.0M. Gillespie observed that the addition of NaCl to sulphide depilatories represses both the swelling of the fibres and the depilatory action. The lesser attack on keratin observed with high concentrations of keratin-dispersing agents could be attributed to slower diffusion caused by repression of swelling.

V. ACKNOWLEDGMENTS

The authors are indebted to Mr. W. G. Crewther for the cortical cells, to Dr. D. H. Simmonds for running chromatograms for the identification of amino acids in various fractions, and to Mr. E. F. Woods for advice and discussions on the theoretical aspects of the electrophoresis studies.

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THE AMINO ACID COMPOSITION OF KERATINS

II. THE AMINO ACID COMPOSITION OF A KERATIN DERIVATIVE EXTRACTED FROM WOOL WITH ALKALINE THIOLYCOLLATE SOLUTION

By D. H. SIMMONDS*

[*Manuscript received July 7, 1954*]

Summary

The amino acid composition of a protein component of Merino 64's quality wool, which moves as a single peak on electrophoresis in alkaline thioglycollate solutions, has been determined. The results, which are summarized in Table 1, show that the purified protein fraction contains more aspartic acid, glutamic acid, leucine, lysine, and amide nitrogen, and less cystine, proline, serine, and tryptophan, than the parent wool from which it was extracted.

I. INTRODUCTION

Gillespie and Lennox (1953, 1955) have demonstrated the presence of several protein constituents in dispersions of wool in alkaline potassium thioglycollate solutions. They have described the extraction of a protein constituting the main component of the dispersion, which moves as a single peak on electrophoresis in alkaline buffers containing potassium thioglycollate.

In the present paper, preliminary data on the amino acid composition of this protein are presented, and the results are compared with a similar amino acid analysis of whole Merino 64's quality wool (Simmonds 1954a).

II. EXPERIMENTAL

(a) Preparation of the Sample for Analysis

The protein preparation was extracted from Merino 64's virgin wool or dry combed top according to the procedure of Gillespie and Lennox (1955). The fraction containing the main component (fraction "F" of Gillespie and Lennox 1955), was checked to ensure the presence of only a single peak on electrophoresis at pH 11.0 under the conditions described by them. The pH of this fraction was then adjusted to 5.0 to precipitate the protein, and the precipitate dissolved in one-tenth the original volume of 0.1M potassium thioglycollate at pH 10.5 and dialysed against running tap water. The solution was freeze-dried and the protein obtained as a fluffy white powder, which was difficult to handle because of its electrostatic charge.

Two samples of approximately 50 mg were placed in test tubes containing 10 ml of twice-distilled constant-boiling HCl. The tubes were evacuated and

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sealed. Hydrolysis at 110°C was carried out for 22 and 70 hr respectively, at the end of which time the tubes were opened and the contents evaporated *in vacuo*. Each was made up to 10 ml with water, and the nitrogen content determined by the micro-Kjeldahl technique. Samples of each hydrolysate were then pipetted into polythene cups and evaporated over KOH and P₂O₅ to remove HCl before application to the "Dowex 50" columns. The sample size was adjusted so that the loading was approximately 0.3 mg nitrogen on the 100-cm "Dowex 50" columns, and 0.6 mg nitrogen on the 15-cm "Dowex 50" columns.

Cystine was determined by oxidation to cysteic acid using performic acid, according to the method of Schram, Moore, and Bigwood (1954). The oxidized and hydrolysed samples, after evaporation nearly to dryness twice with distilled water in a rotary evaporator of the type described by Craig, Gregory, and Hausmann (1950), were made up to 10 ml with distilled water. After determination of the nitrogen content, samples were placed in polythene cups and evaporated to dryness over KOH and P₂O₅.

(b) Analytical Procedure

The ion exchange chromatographic and ninhydrin colorimetric procedures of Moore and Stein (1948, 1951) were used to estimate individual amino acids. Preparation of the columns and solutions and conduct of the analyses were as previously described (Simmonds 1954*a*), except that a magnetic balance (Simmonds 1954*b*) was used for the collection of 1-ml fractions.

Tryptophan and proline were determined by methods previously described (Simmonds 1954*a*). Cysteic acid was separated from the other amino acids in the oxidized protein samples on a 100-cm "Dowex 50" column equilibrated with pH 3.30 citrate buffer. A preliminary run on the unoxidized protein component showed complete absence of ninhydrin-positive material before the emergence of aspartic acid. With the oxidized samples cysteic acid emerged unretarded as a symmetrical peak at the buffer front (about tube 35). Two samples of purified cystine treated in a manner identical with that of the protein component gave an average overall recovery of 96.47 ± 0.12 per cent., and the cystine figure obtained from the protein hydrolysate was accordingly corrected for decomposition during oxidation and hydrolysis by this factor, as suggested by Schram, Moore, and Bigwood (1954). There is some evidence, however (Thompson, personal communication), that small changes in the conditions of oxidation and hydrolysis affect the recovery of certain amino acids, and the cystine figure quoted in Table 1 is to be regarded as tentative.

We have been unable to estimate the proportion of methionine present in the preparation since the performic acid oxidation product, methionine sulphone, appears at about tube 110, incompletely separated from serine. Because of the small amount of methionine present in the protein, we were unable to detect it in the unoxidized samples, and so far insufficient material has been available to allow us to use the conventional Baernstein procedure (Baernstein 1932, 1936).

III. RESULTS

Elution curves for 22-hr hydrolysates of oxidized and unoxidized protein samples are compared in Figure 1, while Table 1 summarizes the results of these analyses together with data derived from 70-hr hydrolysates of unoxidized material. Figures for the tryptophan, proline, nitrogen, and sulphur contents are also included.

TABLE 1

AMINO ACID COMPOSITION OF HYDROLYSATES OF A KERATIN DERIVATIVE EXTRACTED FROM MERINO 64's QUALITY WOOL

Amino acid nitrogen expressed as a percentage of total nitrogen. Nitrogen: 15.26 ± 0.05 per cent.
Sulphur: 2.55 per cent.

Treatment	Unoxidized Samples						Oxidized Samples			Averaged or Adjusted Results. Data of Columns 2 and 3
	22-Hr Hydrolysate			70-Hr Hydrolysate			22-Hr Hydrolysate			
Amino Acid	No. of Analy- ses	Mean	S.E.	No. of Analy- ses	Mean	S.E.	No. of Analy- ses	Mean	S.E.	
1		2			3			4		5
Alanine	3	4.22	0.22	3	4.03	0.22	2	4.62	0.27	4.13
Arginine	2	21.12	0.40	3	19.87	0.32	2	18.40	0.40	20.50
Aspartic	4	5.68	0.34	3	5.09	0.40	2	5.70	0.48	5.39
Amide N	3	11.56	0.09	3	11.54	0.09	2	12.92	0.11	11.56
Cystine							2	4.33	0.03	4.33*
Glutamic	3	10.82†	0.09	3	9.10†	0.09	2	10.76†	0.11	11.72
Glycine	3	5.27	0.28	3	4.87	0.28	2	5.70	0.34	5.07
Histidine	2	1.42	0.07	3	1.32	0.06	2	0.24	0.07	1.37
Isoleucine	4	2.24	0.10	3	2.25	0.11	2	2.38	0.14	2.25
Leucine	4	6.01	0.14	3	5.95	0.17	1	5.39	0.29	5.98
Lysine	3	5.03	0.14	3	4.46	0.14	2	4.46	0.17	4.65
Phenylalanine	4	1.72	0.13	3	1.68	0.15	—	—	—	1.70
Proline	1	3.66								3.66
Serine	4	6.47	0.08	3	6.12	0.09	2	5.98	0.11	6.66
Threonine	3	4.44	0.13	3	3.34	0.13	2	3.54	0.16	5.05
Tryptophan‡	4									0.71‡ ±0.03
Tyrosine	4	2.46	0.12	3	2.10	0.13	—	—	—	2.28
Valine	3	3.55	0.18	3	3.91	0.18	2	3.86	0.21	3.74

* From data of column 4.

† Corrected for pyrrolidone carboxylic acid formation (Moore and Stein 1951).

‡ Method of Goodwin and Morton (1946).

Data derived from the figures in column 5 of Table 1 are summarized in Table 2. This shows the weight of each amino acid and amino acid residue obtainable from 100 g of the protein, together with data on the number of

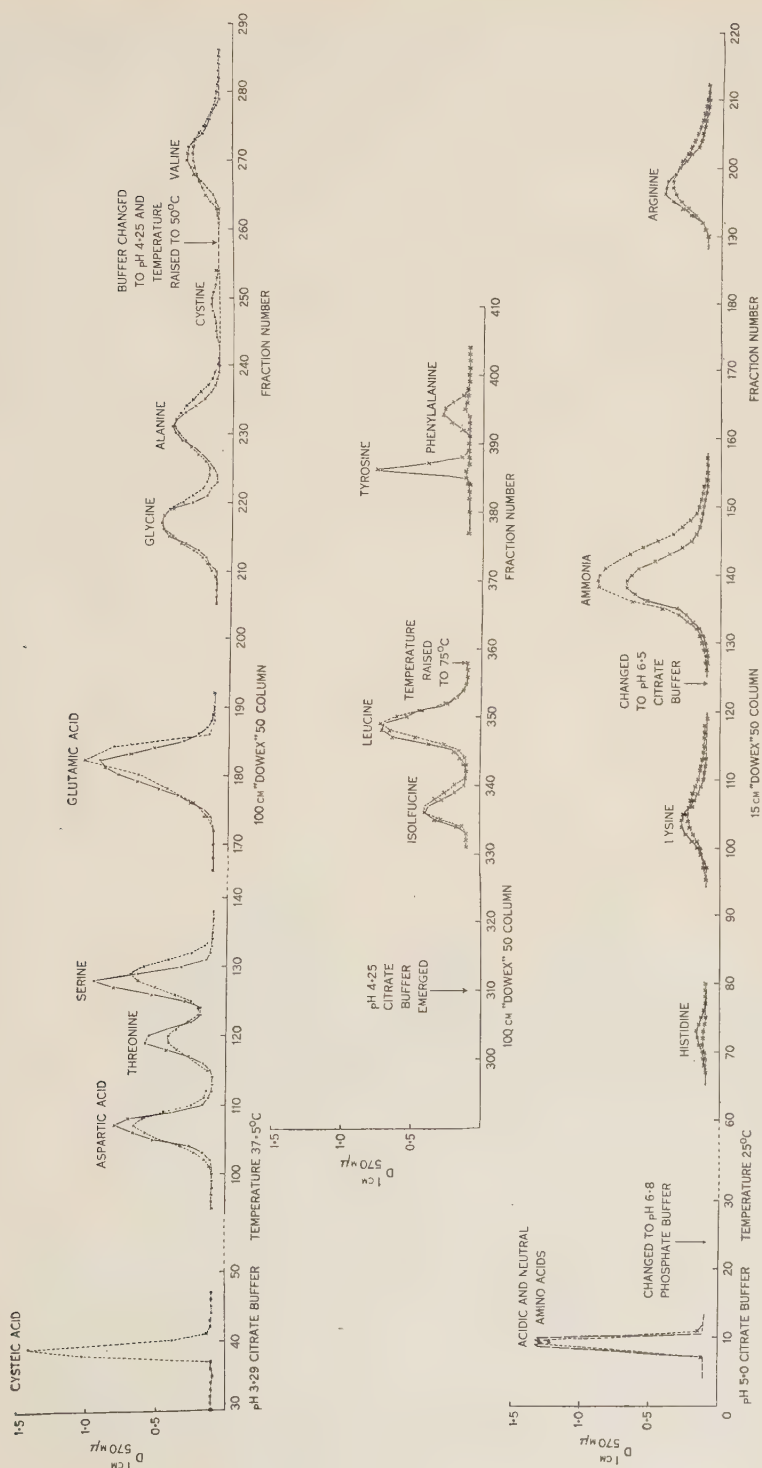


Fig. 1.—Comparison of 22hr hydrolysates of a keratin derivative. x—x. Unoxidized. x-x-x-x-x. After oxidation with performic acid by the method of Schram, Moore and Bigwood (1954).

residues of each amino acid contained in an assumed molecular weight of 15,000, which is probably close to the true value as indicated by the studies of Harrap (1955). The figures used in the calculations for serine, threonine, and glutamic acid were obtained by extrapolation to zero time of hydrolysis as suggested by Smith and Stockell (1954).

TABLE 2

AMINO ACID COMPOSITION OF A KERATIN DERIVATIVE EXTRACTED FROM MERINO 64's QUALITY WOOL

Derived from data in column 5 of Table 1

Amino Acid	Wt./100 g Dry Protein	Wt. Residues/100 g	No. of Residues per M.W. 15,000
Alanine	4.01	3.20	6.75 \approx 7
Arginine	9.72	8.71	8.19 8
Aspartic	7.81	6.75	8.81 9
Amide	2.02	2.02	18.90 19
$\frac{1}{2}$ Cystine	5.67	4.82	14.16 14
Glutamic	18.79	16.49	19.16 19
Glycine	4.14	3.15	8.29 8
Histidine	0.77	0.68	0.75 1
Isoleucine	3.21	2.77	3.68 4
Leucine	8.54	7.37	9.78 10
Lysine	3.70	3.24	3.80 4
Phenylalanine	3.86	3.44	2.78 3
Proline	4.59	3.87	5.98 6
Serine	7.62	6.33	10.89 11
Threonine	6.59	5.59	8.26 8
Tryptophan	0.79	0.72	0.58 1
Tyrosine	4.50	4.05	3.73 4
Valine	4.76	4.03	6.10 6

IV. DISCUSSION

In view of recent reports of the destruction of amino acids during acid hydrolysis (Hirs 1954; Smith and Stockell 1954; Smith, Stockell, and Kimmel 1954) we have prepared and analysed one 22-hr and one 70-hr hydrolysate of the purified wool protein fraction, together with 22-hr hydrolysates of two samples which had been subjected to performic acid oxidation. An analysis of the data in Table 1 has shown that in the 22-hr and 70-hr hydrolysates of the unoxidized protein, the glutamic acid figures differ significantly at the 1 per cent. level, while those for serine and threonine differ at the 5 per cent. level. The remainder, including those for aspartic acid and arginine, are not significantly different at the 5 per cent. level. Only the figures for glutamic acid, serine, and threonine have therefore been linearly extrapolated to zero time of hydrolysis, while the remaining figures have been averaged. These results are recorded in the final column of Table 1. A puzzling feature of the results is that there is no corresponding increase from 22 to 70 hr, in the figures for am-

monia nitrogen, as has been reported by Hirs (1954), Smith and Stockell (1954), and Moore (personal communication).

The effect of performic acid oxidation shows that this treatment has a significant effect on the serine, threonine, tyrosine, phenylalanine, and histidine contents. Consistent with the destruction of these amino acids is the corresponding increase in the ammonia nitrogen figure. Previous authors (Toennies and Homiller 1942; Hirs 1954; Smith and Stockell 1954) have differed on what amino acids are destroyed, and it would appear to depend on the conditions of oxidation and on the amino acid sequence adjacent to the labile amino acid. Hirs (1954) noted that on oxidation of ribonuclease A at -10°C with preformed performic acid, no effect on the amino acid composition was observed other than the conversion of cystine to cysteic acid and methionine to its sulphone. Smith and Stockell (1954), using different conditions of oxidation, reported partial destruction of phenylalanine and almost complete destruction of tyrosine. Blackburn and Lowther (1951) mentioned the possibility of destruction of threonine, in agreement with our results, which also confirm those of Toennies and Homiller (1942) and Sanger and Tuppy (1951) regarding the stability of

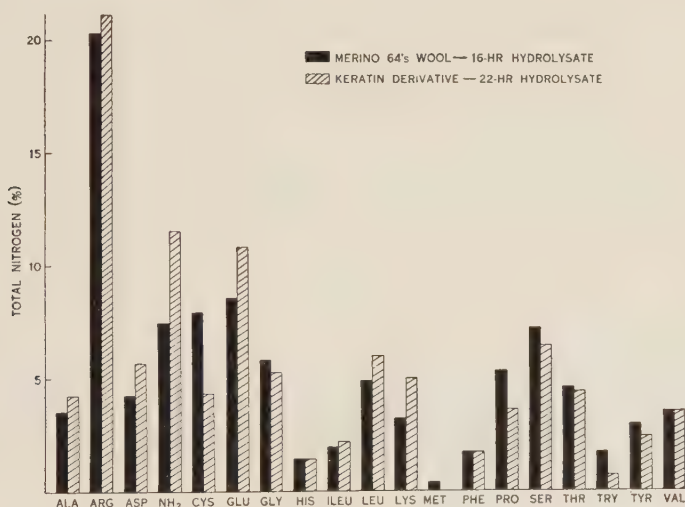


Fig. 2.—Comparison of the amino acid composition of a keratin derivative with that of the Merino 64's wool from which it was extracted. Figures represent percentages of total nitrogen.

the acidic and aliphatic neutral amino acids. Further work will be necessary to decide whether adequate correction factors can be applied to eliminate the need for a duplicate series of determinations on both oxidized and unoxidized samples, but from the present results it does not appear that this approach will be successful.

Differences in the amino acid composition of the 22-hr hydrolysate of the pure protein component and of a 16-hr hydrolysate of Merino 64's quality wool (taken from Table 3, Simmonds 1954a) are most readily seen by reference to the

block graph of Figure 2. The figure quoted in this for cystine (4.33 per cent.) is that obtained from the samples of wool protein which had been oxidized with performic acid. The difference of 6 hr in the hydrolysis times of the whole wool and the purified protein component would be expected from the results summarized in Table 1 and discussed above, to slightly affect the relative amounts of glutamic acid, serine, and threonine present in the two samples. This effect is small and it is considered that the graph clearly shows that the purified protein component appears to contain more of the acidic amino acids, aspartic and glutamic, and more leucine, lysine, and ammonia nitrogen, but less cystine, proline, serine, and tryptophan than the parent wool from which it was derived. This is in accord with recent theories on the structure of the cortex of Merino wool fibres (Horio and Kondo 1953; Mercer 1953; Fraser and Rogers 1953; Fraser, Lindley, and Rogers 1954), where it is assumed that the "S" segment in the terminology of Fraser and Rogers (1953), containing fewer cystine disulphide cross linkages, is more susceptible to chemical attack.

In contrast to the nitrogen, the sulphur content has not been entirely accounted for. The sulphur figure (2.55 per cent.) exceeds that calculated from the cystine content (1.51 per cent.) by 1.04 per cent. The methionine contribution to this difference is likely to be small, and it is assumed that the remainder is derived from thioglycollic acid which is bound to some of the cysteine residues to form the mixed disulphide. No evidence was found during the analysis of the unoxidized protein samples, of peaks which could have been due to a mixed disulphide of cystine and thioglycollic acid, but it is possible that such a combination would have been ruptured during the hydrolysis with 6N HCl and reformed as cystine and the oxidized form of thioglycollic acid.

V. ACKNOWLEDGMENTS

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THE MOLECULAR WEIGHT AT THE AIR-WATER INTERFACE OF SOME KERATIN DERIVATIVES EXTRACTED FROM WOOL

By B. S. HARRAP*

[Manuscript received July 2, 1954]

Summary

Using the monolayer technique, number-average molecular weights have been determined for a series of extracts of wool prepared by successive treatments with alkaline sodium thioglycollate. The molecular weights of these extracts have been discussed in relation to their electrophoretic patterns. The change in the number-average molecular weight in the successive extracts has been correlated with the presence of certain electrophoretic components. The possibility of extraction of lipoidal or other non-protein material from the cortical cell walls is discussed. A reversible dissociation of the major electrophoretic component at high pH was observed.

I. INTRODUCTION

The development, by Guastalla (1939, 1951), of methods for measuring the surface pressure of amphipathic molecules spread at the air-water interface at very low surface concentrations has made it possible to determine the molecular weight of large molecules.

Subsequent work by Bull (1950) has shown that the surface pressure is thermodynamically equivalent to a two-dimensional osmotic pressure, and that the variation of surface pressure (π) with area (A) may be represented by an equation

$$\pi (A - A_0) = n R T = NW/M \cdot RT, \quad \dots \quad (1)$$

where A_0 is a factor representing the "co-area" of the film molecules, N is Avogadro's number, M the molecular weight of the film molecules, W the weight of material spread at the interface, and R and T the gas constant and absolute temperature respectively. Since W/A is the surface concentration, the analogy between equation (1) and the familiar three-dimensional osmotic pressure-concentration equation is apparent. Thus, by measuring the variation of surface pressure with surface concentration, the molecular weight of the spread molecules may be determined.

However, it has not always been realized that equation (1), in common with its three-dimensional analogy, holds only at low surface concentrations where interaction between the film molecules is negligible; otherwise higher-power terms in A must be introduced into equation (1). To illustrate this point, let us consider a typical protein molecule which is thought to spread in the β configuration (Bull 1947). From X-ray data, the mean side-chain length

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is of the order of 10 Å for most proteins in this configuration and the unit repeat distance per residue along the polypeptide chain is about 3.5 Å. Thus, if the protein molecule is oriented at the interface with one of its side-chains parallel to the water surface the area occupied by that residue will be about 35 Å². If any other protein molecule approaches within that area interaction will occur. Taking the mean residue weight of a typical protein as 110, this area corresponds to

$$\frac{35 \times 6.06}{110} = 1.9 \text{ m}^2/\text{mg}.$$

Hence, if the protein film is compressed to areas below this figure intermolecular interaction is possible. Several earlier workers have reported measurements at areas as low as 1 m²/mg: the molecular weights calculated from these observations must therefore be treated with reserve.

This paper reports measurements of the molecular weights of some of the wool protein extracts prepared and described by Gillespie and Lennox (1955). All measurements were made at areas greater than 2 m²/mg where it is likely that intermolecular interaction is very small and that equation (1) holds.

II. EXPERIMENTAL

(a) *Surface Balance*

The surface balance used was basically of the conventional Adam-Langmuir horizontal-float type, but incorporating the modifications suggested by Few and Schulman (1953) (q.v.), together with the following new features:

(i) The light aluminium framework which follows the movement of the float was supported by two agate knife-edges bearing on two agate flats.

(ii) A movable brass bob mounted on top of this framework was used to vary the sensitivity of the balance.

(iii) By means of an externally controlled rider mechanism, similar to that on a conventional beam balance, the sensitivity of the balance could be checked during a series of measurements without disturbing the temperature equilibrium inside the balance case.

At the setting of maximum sensitivity of the balance, surface pressure changes of about 0.3 millidyne/cm could be readily estimated. For maximum reproducibility it was found essential to stand the balance on a vibration-free pier and to protect it from draughts.

(b) *Spreading Solutions*

The protein solutions were spread using an "Aglā" micrometer syringe which could be manipulated from outside the balance case (Cheeseman 1952). The surface concentration was varied by keeping the surface area constant and adding successive volumes of the protein solution. Even at the highest surface concentration the pressures involved were so low that there was no likelihood of the formation of the so-called *B*-films of Joly (1939).

Stock solutions of the wool protein were prepared by dissolving the freeze-dried extracts, supplied by Gillespie and Lennox (1955), in phosphate buffer of ionic strength 0.05 which was also 0.001M with respect to thioglycollic acid.

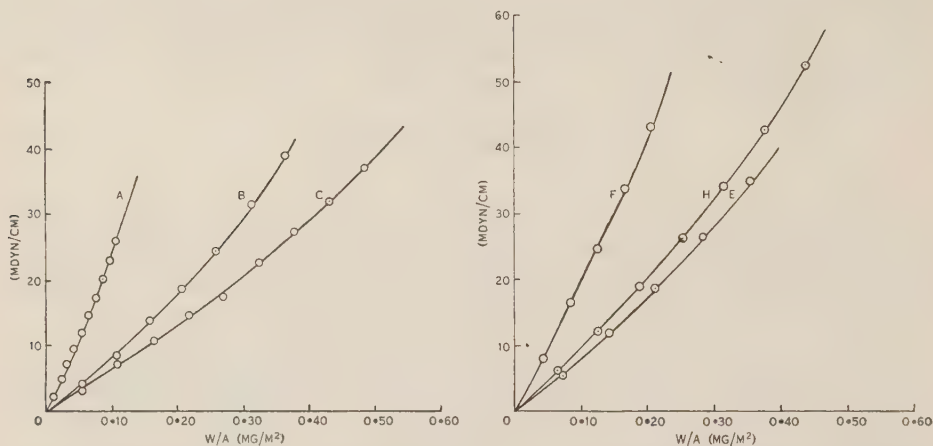


Fig. 1.—Surface pressure: surface concentration curves for alkaline thioglycollate extracts of wool, spread from undialysed solutions. Subsolution pH 6.4.

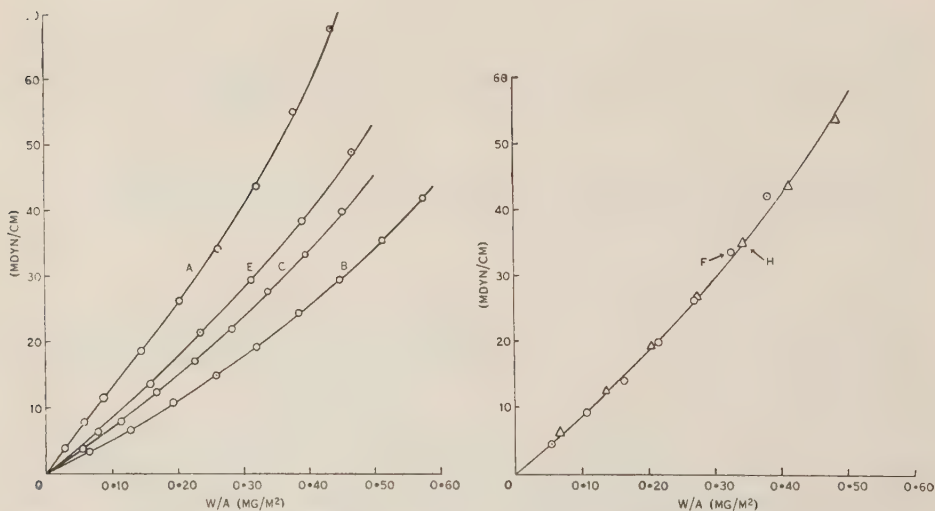


Fig. 2.—Surface pressure: surface concentration curves for alkaline thioglycollate extracts of wool, spread from exhaustively dialysed solutions. Subsolution pH 6.4.

The concentrations of these stock solutions were determined by Kjeldahl nitrogen estimations and solutions for spreading prepared by quantitative dilution with the same buffer to give *c.* 0.01 per cent. solutions.

The protein was spread on a subphase consisting of 0.9M KCl to which phosphate buffer salts were added to give the required pH and ionic strength $\Gamma/2 = 1$. On this subphase complete spreading (checked as suggested by Bull

1947) occurred spontaneously, without the addition of any extraneous materials, such as alcohols, to assist spreading.

(c) Materials

All water used was doubly distilled, the second time from alkaline permanganate in an all-glass still. Buffer salts and sodium chloride were of analytical reagent quality and where possible were ignited at 500°C to remove surface-active impurities. All solutions were allowed to stand for 2 hr before use to enable surface-active impurities to accumulate at the surface. The solution to be used was then siphoned off from below the surface.

TABLE 1
MOLECULAR WEIGHT OF KERATIN EXTRACTS CALCULATED FROM FIGURES 1 AND 2

Extract	pH of Extraction	Molecular Weight ($\times 10^{-3}$)	
		Before Dialysis*	After Dialysis†
A	10.5	11	22.9 \pm 1
B	10.5	32	46.8 \pm 1
C	10.5	38	37.7 \pm 1
E	10.5	32	29.6 \pm 1
F	12.3	12.5	32.5 \pm 1
H	12.3	31	32.7 \pm 0.6

*Since these values are for purposes of comparison only they have been simply calculated from plots of FA v. F .

† These values have been calculated by the method of least squares.

III. RESULTS AND DISCUSSION

Curves of π v. W/A for the extracts A, B, C, E, F, and H are shown in Figures 1 and 2. These extracts were prepared by Gillespie and Lennox (1955) by successive extractions of wool with alkaline thioglycollate.

The freeze-dried extracts were dissolved in phosphate-thioglycollate buffer of pH 10.4, ionic strength $I/2 = 0.05$, and spread on a subphase of pH 6.4. Figure 1 gives the results using spreading solutions prepared from undialysed stock solutions. The results in Figure 2 were obtained using spreading solutions prepared from stock solutions, 25 ml of which had been previously exhaustively dialysed through "Visking" sausage casing against eight successive 200-ml changes of the same phosphate-thioglycollate buffer over a period of 4 days. The molecular weights obtained from these curves are shown in Table 1.

Considering first extracts A-E (extracted at pH 10.5), the molecular weights* in Table 1 show that extract A, which was found by Gillespie and Lennox (1955) to comprise several electrophoretic components, contains a con-

* The molecular weights obtained by this technique are number-average values, similar to those obtained from osmometry.

siderable quantity of low-molecular-weight material. Some of this is dialysable, indicating a molecular weight of less than 10,000. Extract *B*, which is also electrophoretically polydisperse, contains some dialysable material, together with a very high molecular weight component. Extracts *C* and *E*, on the other hand, show no significant change in molecular weight on dialysis and their electrophoretic patterns show that components 1 and 4 have virtually disappeared. It therefore seems reasonable to identify these two components with the low-molecular-weight dialysable material in extracts *A* and *B*. Indeed, Gillespie and Lennox (1955) have noted that the relative proportions of the subcomponents comprising component 1 vary with the preliminary equilibrium dialysis time prior to electrophoresis. It is possible that component 1 or 4 may contain non-protein material, since Gillespie and Lennox (1955) observed very little effusion of nitrogenous material on dialysis of extracts *A* and *B*. The discrepancy between this and the large change in number-average molecular weight on dialysis may arise from the presence of low-molecular-weight, but highly surface-active, lipoidal material in the fibre extract. This hypothesis is supported by the electrophoresis diagram for the supernatant liquid obtained after precipitating the protein in extract *B* with acid; this shows a broad, flat band, uncharacteristic of protein solutions.

Table 1 also shows a decrease in the number-average molecular weight from extracts *B-E*, indicating a decrease in the proportion of high-molecular-weight material. The decrease in the range of molecular weights present on passing from *B* to *E* is paralleled by the enhancement of electrophoretic homogeneity, resulting in the emergence of component 2 as virtually a single electrophoretic peak in extract *E*. It is likely therefore that this component has a molecular weight in the vicinity of 30,000 at pH 6.4, whilst component 3, which persists to extract *D* in the electrophoretic diagrams, is probably of high molecular weight and thereby responsible for the high number-average molecular weights observed for extracts *B* and *C*.

Turning to extracts *F* and *H*, prepared by raising the pH to 12.3, Table 1 shows a large increase in the molecular weight of the *F* extract on dialysis, suggesting that raising the pH results in the extraction of a further quantity of low-molecular-weight, dialysable material. The molecular weight of the dialysed extract is, however, not significantly different from that for extract *E* and the electrophoretic diagrams show that the two extracts contain the same electrophoretic component. We must therefore look for the origin of the low-molecular-weight material in the undialysed sample. A possible explanation is suggested by the rate of extraction of the major electrophoretic component 2. Gillespie and Lennox (1955) have found that very small amounts are extracted at pH 10.5 with each successive treatment, the process seeming to be one of diffusion. On raising the pH to 12.3, however, a much larger quantity is extracted, suggesting that there has been a marked change in the barrier to diffusion. It seems likely that the low-molecular-weight material in the undialysed extract *F* may be the breakdown products of this diffusion barrier. For this extract also Gillespie and Lennox (1955) observed very little dialysable nitro-

genous material. Thus the large change in molecular weight on dialysis seems again to be due to non-protein material. It is known that medulla cells of hair contain considerable quantities of phospholipid (Stoves 1946). If the cortical cell walls contain similar material, possibly in the form of a lipoprotein complex, one would expect the low-molecular-weight lipoidal products from the breakdown of these walls to have a profound effect on the number-average molecular weight of the protein released from within the cells. It is unlikely that the low-molecular-weight material could be a breakdown product of the major component itself as a result of the increased pH, since extract *H*, which has been

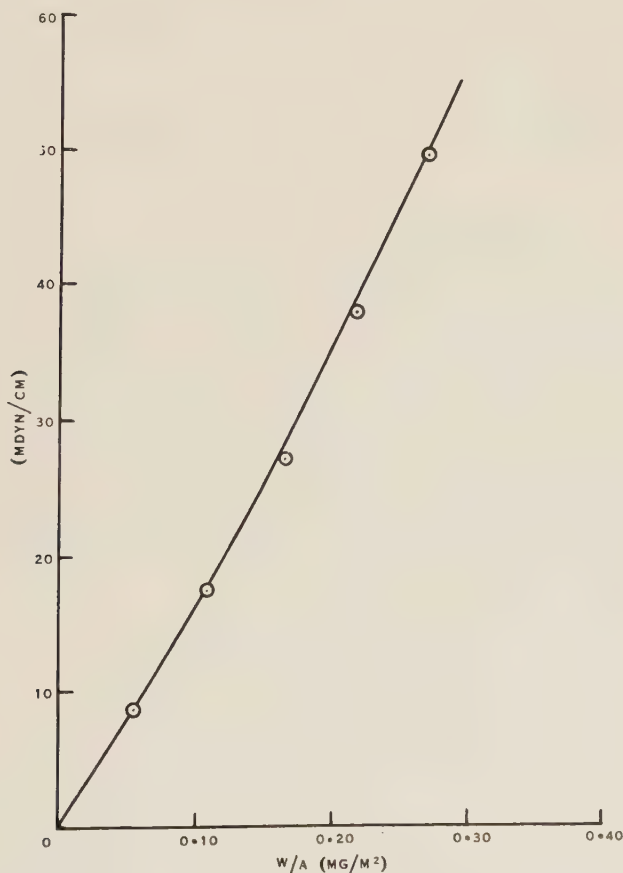


Fig. 3.—Surface pressure: surface concentration curve for extract *F*, spread from exhaustively dialysed solution on pH 9.4 subsolution.

subjected to the same conditions, shows no significant difference between the molecular weights of the dialysed and undialysed samples. Moreover, the molecular weight of extract *H* is not significantly different from those of dialysed extracts *E* and *F*. It appears that in these three solutions we are dealing with a molecule which, either by itself or in combination, is a major component

of the wool fibre. The electrophoretic diagram of extract *H* shows, in addition to the major component, a faster-moving spike; since no significant change in molecular weight occurs, this is apparently due to a change in the molecular configuration of the protein.

A few preliminary experiments have also been made to determine the effect of pH of both the spreading solution and the subphase on the molecular weight. First, extract *F* was dialysed against phosphate-thioglycollate buffers of pH 8.1, 10.4, and 11.5, and the resulting solutions spread on a pH 6.4 subphase. There was no significant difference between the $\pi v. W/A$ curves for these three solutions and hence between the molecular weights calculated from them (32,000, 30,000, and 31,000 respectively). The pH 10.4 solution was also spread on subphases of pH 2.1 and 9.4. On the former a molecular weight of 29,000 was estimated, again an insignificant change, but on the pH 9.4 subphase the $\pi v. W/A$ curve shown in Figure 3 was obtained, leading to a molecular weight of 16,000. This suggests a dissociation of the major electrophoretic component as the pH is raised. It appears that the state of aggregation of the *spread* molecule is a function of the pH of the subphase rather than of the solution from which it is spread. Therefore, if the states of aggregation in both the surface phase and in the bulk phase are the same at the same pH, we must conclude that the dissociation of the molecule is reversible since a solution of high pH spread on a subphase of neutral pH gives a surface film of "dimer."

Friend and O'Donnell (1953), from osmotic pressure measurements, obtained a value of 15,000 for the molecular weight of an extract of wool by urea-bisulphite at pH 8. It is tempting to speculate whether the same major component is being dealt with in each case. If we postulate a "dimeric" reduced molecule of molecular weight *c.* 30,000 at neutral pH, the two halves being held together by hydrogen bonds, then we should expect the cleavage of these bonds either by concentrated urea solution or by increasing the pH of the solution.

IV. ACKNOWLEDGMENTS

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THE SURFACE STRUCTURE OF WOOL AND ITS COMPONENTS REVEALED BY METAL SHADOWING

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[*Manuscript received October 11, 1954*]

Summary

Gold shadowing reveals considerable details of surface modifications in damaged wool fibres when viewed by transmitted light in the optical microscope. The method is more rapid and less likely to introduce artefacts than the replica techniques currently employed in electron microscopy. New evidence of the nature of the inner surface of the cuticle is also described.

I. INTRODUCTION

A number of electron microscope investigations of surface modifications in damaged wools have been described in which replica techniques were employed (Swerdlow and Seeman 1948; Makinson 1950; Mercer and Roadnight 1950; Elliot and Manogue 1952; Mercer 1953). The direct examination of fibre surfaces in the electron microscope is not possible and little detail is seen by examination with transmitted light in the optical microscope. Dempster and Williams (1946) and Scott and Wyckoff (1949) have stressed the use of gold shadowing as a method of revealing surface texture in the optical microscope and we have recently pointed out the value of this technique in studying the fine histology of wool fibres (Fraser and Rogers 1954*a*). We have now examined wool fibres subjected to a variety of chemical and mechanical treatments and find that details of surface modifications may readily be seen when the wool fibres are gold-shadowed and viewed by transmitted light in the optical microscope.

II. PREPARATION OF SPECIMENS

Selected samples of Merino, Crossbred, and Corriedale wools were solvent-scoured, washed in distilled water, and dried to room humidity. Small staples or individual fibres were then subjected to the following treatments and shadowed with gold at an angle of 35° in the manner already described (Fraser and Rogers 1954*a*).

Stretched wool.—Individual fibres of Corriedale 56's quality were slowly extended 50 per cent. in steam and others 100 per cent. in boiling water with a simple extensometer.

Tryptic digestion.—Corriedale wool (5 g) was incubated for 4 days at 40°C in 500 ml of 1 per cent. crude trypsin (pH 8.5) in the presence of 0.01 per cent. merthiolate as antiseptic, washed in distilled water, and air-dried.

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Chlorine and bromine.—Merino 64's quality wool was treated with freshly prepared saturated chlorine or bromine water in the ratio 1 g/100 ml for 15 min, rinsed twice with distilled water, shaken for 15 min with distilled water to remove epicuticle, and finally air-dried.

Sulphuric acid.—Standard commercial top of Merino 64's quality was soaked for 15 min in 10 per cent. w/v sulphuric acid, squeezed out, and dried for 1 hr in a stream of hot air at 100°C.

Cetyl sulphonic acid.—Merino 64's quality wool was incubated with 0.05M cetyl sulphonic acid (pH 2) at 65°C for 6 days, washed, treated with 0.01N ammonium hydroxide for 15 min, and air-dried. This treatment has been shown to disperse the S (alkali-susceptible) component of the bilateral cortex (Fraser, Lindley, and Rogers 1954).

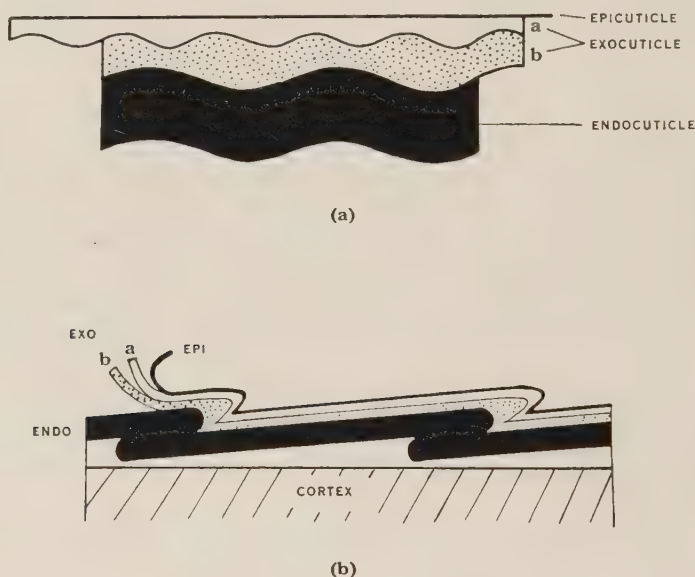


Fig. 1.—(a) The fine structure of the surface layers of wool in transverse section (Lindberg *et al.* 1949; Lagermalm 1954).
(b) The distribution of these layers in longitudinal section (Fraser and Rogers 1954b).

S-carboxymethyl wool.—Merino 64's quality wool was repeatedly reduced with 0.4M sodium thioglycollate, pH 5.6, and alkylated with 0.5 per cent. iodoacetic acid in buffer at pH 7.2, washed, and dried (O'Donnell 1954).

III. RESULTS

Current theories of the fine structure of the surface layers of wool are summarized in Figure 1 (Lindberg *et al.* 1949; Lagermalm 1954; Fraser and Rogers 1954b) and the detail revealed by gold shadowing will be described in relation to these component layers.

(a) Fibre Surfaces

(i) *Extended Wool*.—Examples of the effect on the cuticle when wool fibres are extended in steam are shown in Plate 1, Figures 2, 3, 5, and 6. No typical damage for a given percentage extension was observed as the strain was localized to some extent. In general, however, the inter-scale tip distance is increased, as compared with unstretched fibres (Plate 1, Figs. 1 and 4), owing to either slippage or extension of the cuticle, or both. The appearance of Merino fibres, in which the scale overlap is small, after 100 per cent. extension in steam suggests that the scale substance possesses a long-range extensibility similar to that of the cortex. At 50 per cent. extension (Plate 1, Fig. 2) the scale tips of Corriedale fibres are seen to detach from the underlying scales and protrude from the general surface of the cuticle. This feature is clearly seen in Plate 1, Figure 5, which shows the profile of such a fibre. At 100 per cent. extension (Plate 1, Figs. 3 and 6) a considerable portion of the scale becomes detached, and large flaps protrude from the surface of the fibres.

(ii) *Trypsin-treated Wool*.—It is well known that the cuticle of wool is extensively damaged by the action of crude trypsin, and Plate 2, Figure 1, shows the surface of a fibre in an advanced stage of degradation. Large areas of individual scales appear to have flaked off, giving a chipped appearance. In this particular case the epicuticle seems to have been detached although at other points on the fibre it appeared to be intact, producing a similar appearance to that noted by Mercer (1953).

(iii) *Chlorine and Bromine*.—These reagents cause considerable degradation of the outer layers of the cuticle, and Plate 2, Figure 2, shows the action of chlorine on the scale tips and folds of epicuticle which have dried down to give a "doubling" of the scale edges. In other fibres, where the epicuticle had been detached, the striations of the external scale layers were visible as reported by Swerdlow and Seeman (1948), Elliot and Manogue (1952), and Mercer (1953). The action of bromine on the scale tips is shown in Plate 2, Figure 3, where the epicuticle has been detached, and the corrugated surface of the endocuticle is revealed.

(iv) *Sulphuric Acid*.—The damage that occurs following sulphuric acid treatment is of interest as this is the reagent utilized in carbonizing. Our observations are in general agreement with those of Mercer (1953), who reported very little change in the appearance of the surface after carbonizing. Even excessive carbonizing produces only slight evidence of surface damage, the fibre of Plate 2, Figure 4, is a typical example showing only an exaggerated prominence of the scale tips and occasional chips in the scale edges.

(v) *Cetyl Sulphonic Acid*.—It will be seen from Plate 2, Figure 5, that the cuticle has been extensively damaged by this treatment and it gives the appearance of a thin membrane, presumably the epicuticle, which has dried down on

a much depleted scale structure. The longitudinal furrows are extremely prominent in this case.

(vi) *S-carboxymethyl Wool*.—Although the fibres in this instance have not been exposed to extremes of pH some surface modification has occurred (Plate 2, Fig. 6). The furrows are similar to those of the bromine-treated fibre of Plate 2, Figure 3, and probably reflect the corrugated surface of the underlying endocuticle. It is probable that this feature arose from differential changes in the outer layers of the cuticle during the reduction process.

(b) *The Component Layers of the Cuticle*

In addition to its value as a method for detecting surface modifications in damaged wool fibres, gold shadowing provides a method of correlating the fine structural detail observed in the electron microscope with the coarse histological features seen in the optical microscope. New evidence of the nature of the component layers of the cuticle is presented in Plate 3, Figure 1, in which the cuticle has been partially degraded by trypsin, and in Plate 3, Figures 2, 3, and 4, in which wool fibres treated with cetyl sulphonic acid have been extracted with ammonium hydroxide.

The main scale fragment in Plate 3, Figure 1, has a smooth outer surface *a*, similar to that of the fibre in Plate 2, Figure 1, and ridges corresponding presumably to the junctions of overlapping scales, are also visible. The inner surface *b* (Plate 3, Fig. 1) of the smaller scale fragment has a furrowed structure of similar periodicity to the endocuticle as depicted in Figure 1. The furrows terminate in a thickened edge which is probably associated with the ridges on the outer surface of the main scale fragment.

Further evidence of a smooth chemically resistant outer layer of the cuticle is seen in Plate 3, Figure 2, although there is some evidence of longitudinal striations in this case. Plate 3, Figure 3, shows the appearance of the inner surface of a cuticle fragment from the same preparation. Various stages of degradation are present in this fragment; at the thickest part *a* the inner surface is comparatively smooth and featureless and appears to be continuous across the inner edge of the adjacent scale. At *b* this layer presents a pock-marked appearance and between *b* and *c* longitudinal furrows similar to those of Plate 3, Figure 1, are seen. The furrowed layer between *b* and *c* is continuous across the inner scale edge but ends abruptly to reveal a thinner striated layer at *c*.

Further evidence of the fact that the inner scale edges project from the inner surface of the cuticle is seen in Plate 3, Figure 4, which depicts a cortical cell isolated from the *H* segment of the bilateral cortex of merino wool by controlled hydrolysis with cetyl sulphonic acid (Fraser, Lindley, and Rogers 1954). The transverse depressions revealed by the shadowing probably correspond to the impressions left by the inner scale edges, that is to say, the cell was located on the periphery of the cortex with its uppermost surface in contact with the inner layer of the cuticle.

IV. DISCUSSION

(a) Fibre Surfaces

It appears, from the specimens that we have prepared, that the examination of gold-shadowed wool fibres by transmitted light in the optical microscope provides a simple and rapid method of assessing surface modification. The resolution of the optical microscope is limited to about $0.3\ \mu$ but structural detail near this limit is readily visible owing to the high contrast obtained. Although the resolving power of the electron microscope is very much greater, the replica technique employed in the preparation of specimens is both tedious and liable to produce artefacts.

The depth of focus of a high numerical aperture, short-focus objective is extremely small and it is difficult therefore to record photographically the details of curved fibre surfaces. This is particularly noticeable in fine wools where the radius of curvature is about $10\ \mu$. This does not detract from the value of the method, however, as with visual observation the objective may be racked up and down in the usual manner.

(b) The Component Layers of the Cuticle

Present-day knowledge of the component layers of the cuticle, summarized in Figure 1, is based upon the examination of scale fragments from grossly degraded fibres in the electron microscope (Mercer and Rees 1946*a*, 1946*b*). A major difficulty, however, in building up a comprehensive picture of the struc-



Fig. 2.—An alternative view of the nature of the component layers in the cuticle of wool seen in transverse section (diagrammatic). *epi*, epicuticle; *exo*, exocuticle; *end*, endocuticle; *r*, resistant outer layer of endocuticle; *f*, fibrillar endocuticle component isolated by enzymic or acid hydrolysis; *c*, endocuticle cementing material.

ture of the cuticle has been the lack of a suitable microscopical method for investigating the relationship between the structural details observed in the electron microscope and the coarser features seen in the optical microscope. This difficulty originates not so much from the limited resolving power of the optical microscope as the low contrast in the comparatively transparent wool fibre components. It is clear from the photomicrographs of Plate 3, however, that this difficulty may be overcome by the use of gold shadowing, as many details of the fine structure of the cuticle, normally only visible in the electron microscope, then become visible in the optical microscope. The transparency

of the wool fibre in the optical microscope is now an advantage as specimen thickness is not severely limited as in electron microscopy and some correlation of fine and coarse histology is possible.

According to Figure 1(*a*) the outer surface of the endocuticle is corrugated, or furrowed, and the furrows run parallel to the fibre axis. This feature is seen in Plate 2, Figure 3, where the exocuticle and epicuticle have been removed by treatment with saturated bromine water (Fraser and Rogers 1954*b*). In fibres partially degraded with trypsin (Plate 2, Fig. 1; Plate 3, Fig. 1) and with cetyl sulphonic acid (Plate 3, Fig. 2) the outer surface of the cuticle appears comparatively smooth. This may be due to the epicuticle masking the nature of the underlying structures, or to the continuous outer layer of the endocuticle which is revealed after oxidation with peracetic acid and extraction with ammonium hydroxide (Fraser and Rogers 1954*c*).

The nature of the inner surface of the enzyme-resistant component of the endocuticle, previously unknown, is clearly seen in Plate 3, Figures 1 and 3, to be similar to that of the outer surface. The thickness of this component of the endocuticle, measured radially from the centre of the fibre, is depicted by Lindberg *et al.* (1949) as constant, but it seems more probable that it may be formed by the coalescence of fibrils *c.* 0.6 μ in diameter. In the intact fibre this component appears to be embedded in a material having less resistance to enzymic or acid hydrolysis (Fig. 2), as the inner surface of the cuticle in both trypsin and cetyl sulphonic acid preparations has a smooth texture where least damage has occurred, e.g. area *a* of Plate 3, Figure 3. In addition, it appears that the inner scale edges project from the inner surface of the cuticle, and this is confirmed by the impression retained in the cortical cell of Plate 3, Figure 4, which, it is presumed, was originally located on the periphery of the cortex with its uppermost surface in contact with the inner surface of the cuticle.

V. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1-3

PLATE 1

Fibre surfaces gold-shadowed from the direction of the tip at an angle of 35° . Magnification $\times 1000$, negative prints.

- Fig. 1.—Untreated fibre, Corriedale 56's quality.
Fig. 2.—Corriedale 56's quality fibre extended 50 per cent. in steam.
Fig. 3.—Corriedale 56's quality fibre extended 100 per cent. in boiling water.
Fig. 4.—Profile of untreated Corriedale fibre.
Fig. 5.—Profile of Corriedale fibre extended 50 per cent. in steam.
Fig. 6.—Profile of Corriedale fibre extended 100 per cent. in boiling water.

PLATE 2

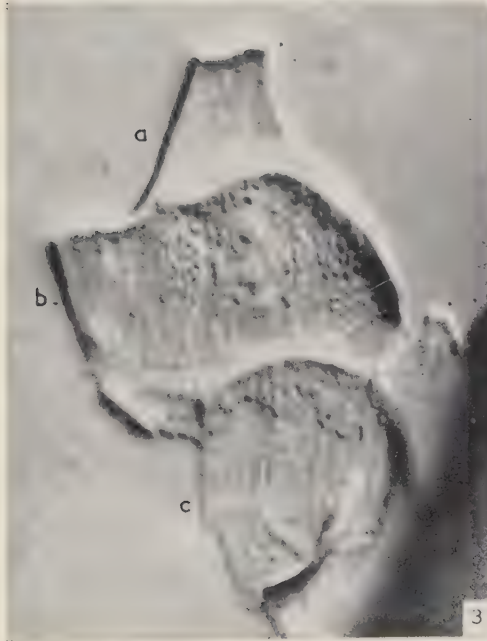
Fibre surfaces gold-shadowed from the direction of the tip at an angle of 35° . Magnification $\times 1000$, negative prints.

- Fig. 1.—Corriedale 56's fibre partially digested with crude trypsin.
Fig. 2.—Merino 64's fibre treated with chlorine (epicuticle intact).
Fig. 3.—Merino 64's fibre treated with bromine (epicuticle and exocuticle detached).
Fig. 4.—Merino fibre treated with 10 per cent. sulphuric acid.
Fig. 5.—Merino 64's fibre treated with cetyl sulphonic acid and ammonia.
Fig. 6.—Merino 64's fibre reduced with thioglycolic acid and oxidized with iodoacetic acid.

PLATE 3

- Fig. 1.—Scale fragment isolated from trypsin-digested wool showing smooth nature of outer surface (*a*), and corrugated inner surface (*b*) of overlying fragment. Magnification $\times 2600$, negative print.
Fig. 2.—Outer surface of scale fragment isolated from wool treated with cetyl sulphonic acid and extracted with ammonia. Magnification $\times 1280$, negative print.
Fig. 3.—Inner surface of scale fragment from the same preparation as Plate 3, Figure 2, showing (*a*) continuous inner cuticle layer; (*b*) partial dispersion of inner cuticle layers; (*c*) complete dispersion of inner cuticle layers. Magnification $\times 1280$, negative print.
Fig. 4.—Cortical cell from the *H* segment of the bilateral cortex from the same preparation as Plate 3, Figure 2. Note transverse depressions left by inner surface of cuticle. Magnification $\times 2000$, positive print.

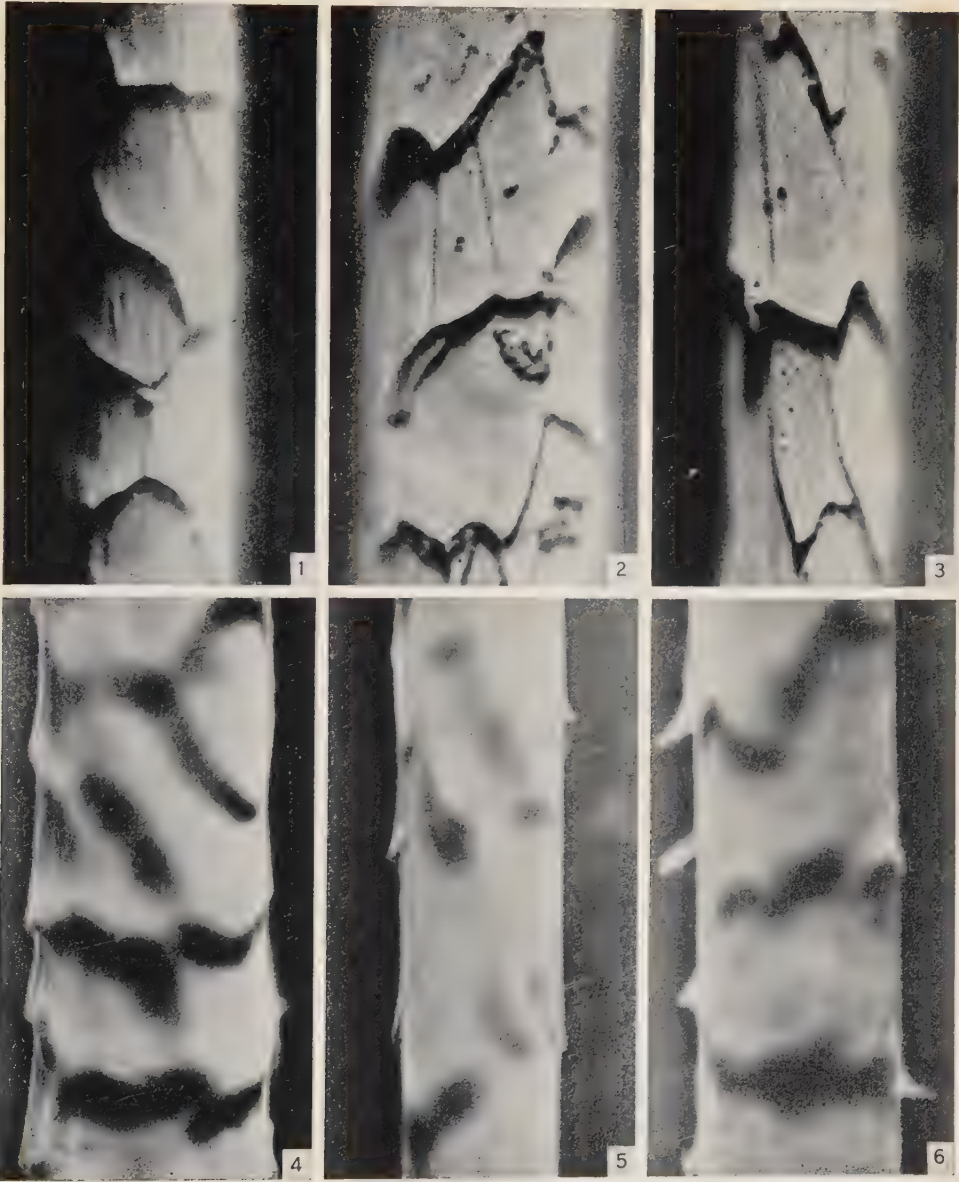
STRUCTURE OF WOOL



STRUCTURE OF WOOL



STRUCTURE OF WOOL



PHYSIOLOGY OF PEA FRUITS

I. THE DEVELOPING FRUIT

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Summary

Pea fruits from two crops were sampled at different times from flowering. Changes in the fresh weight, dry weight, starch, soluble carbohydrate, protein nitrogen, and soluble nitrogen in both seeds and hulls were followed in two seasons and related to the changes in cell volume in the seeds. In one season respiration rates and phosphate, pectin, and ascorbic acid contents were also investigated. The seeds gained more carbohydrate and nitrogen than was lost by the hulls. Starch and protein were synthesized rapidly by the seeds. The increase in starch content in the seeds was followed by a decrease in soluble carbohydrate content, after which the seed ceased to accumulate water. These metabolic changes are discussed in the light of recent biochemical knowledge, and in relation to more detailed biochemical investigations in progress.

I. INTRODUCTION

Germinating seeds have long provided a favourite material for plant biochemists, and have contributed much of our knowledge of the breakdown of protein and complex carbohydrates in plants. The corresponding synthetic processes could logically be studied in ripening seeds. This approach is promising, especially in view of recent advances in the general knowledge of protein and carbohydrate synthesis, but the workers who have studied developing seeds are few compared with those studying germination and the early development of seedlings. A programme of research was therefore initiated to use recent biochemical concepts, such as the importance of phosphate transfer systems in syntheses, in studying the rapid synthesis of carbohydrates, proteins, and other substances in the developing seeds of peas.

The developmental changes in the seed are also of considerable practical interest to the food industry. Beyond a fairly sharply defined stage the pea ceases to give a first class canned or frozen product. Since this stage is reached while the seed is still rapidly increasing in weight, it is important to determine the latest date on which to harvest, so as to obtain the maximal yield of first quality peas. Considerable success has been achieved in the use of physical measurements to determine and predict this optimal harvest date (Lynch and Mitchell 1950), and it is desirable to know whether the stage so defined corresponds to any metabolic landmark in the life of the developing seed.

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(a) Historical

Emmerling (1880, 1887, 1900) made extensive observations, which seem to have been largely neglected by more recent workers, on ripening seeds of the broad bean (*Vicia faba*), and concluded that non-protein nitrogenous substances were translocated to the seeds. Increase of protein in the seed was accompanied by decrease of non-protein nitrogenous substances. Subsequent work, all done with rather large seeds containing much protein, has demonstrated that the relative amount of non-protein nitrogen in the seed decreases during ripening, though the absolute amount may decrease or increase as ripening proceeds. Thus Pfenninger (1909) working with *Phaseolus vulgaris* and Petrie (1911) with *V. sativa* showed increases in the non-protein nitrogen of ripening seeds.

In peas about 10 per cent. of the nitrogen of the mature seed is non-protein (Crocker and Barton 1953) and an absolute decrease in non-protein nitrogen has been observed during ripening (Schulze and Winterstein 1910; Bisson and Jones 1932). Seeds of *Lupinus albus* ripening either on the plant or in detached fruits increased considerably in protein content (Vasiliev 1908; Mothes 1939). Vasiliev found a large transfer of dry matter from hulls to seeds. His data for nitrogen, given only as percentages, not absolute amounts, are difficult to interpret, but suggest little transfer of this element. In isolated seeds he found a marked increase of protein nitrogen, and a concurrent decrease in asparagine nitrogen equivalent to about 70 per cent. of the increase in protein nitrogen. Similarly Pfenninger (1909), with *P. vulgaris*, observed a great increase in total nitrogen per seed and a decrease in total nitrogen per hull. The uptake per seed, however, was approximately twice the loss per hull, so that the great bulk of the nitrogen of the seeds came from other parts of the plant.

The same general trends during the maturation of pea seeds, an increase in protein nitrogen to about 90 per cent. of the total nitrogen and a corresponding decrease in soluble nitrogen, are clearly shown in the results of Boswell (1929). His data, however, are all given as percentages and changes in absolute amounts cannot be calculated from them. Bisson and Jones (1932) recorded at various stages of development the amounts of various constituents in the hull and in the total number of seeds it contained. They found that the contents of nitrogen, sugars, starch, and ash in the hulls all showed an early maximum, and declined later, presumably as a result of translocations to the seeds. For both starch and nitrogen the increase in the seeds of a pod was between four and five times the loss by the hull, demonstrating a considerable transfer of these materials from other parts of the plant to the developing seeds. The exact origin of the materials translocated to the seeds was not determined in these experiments. Vasiliev (1908) noted a decrease during the ripening of the seeds in the total amount of nitrogen in the leaves of plants of *L. albus*, and Emmerling (1900) reported a similar finding with broad beans (*V. faba*).

In all this work it was explicitly or tacitly assumed that nitrogen insoluble in aqueous alcohol is a measure of protein content, no distinction being made between the different types of protein which must be present. Danielsson and

Lis (1952) confirmed with modern techniques the observations of Osborne and Campbell (1898) that mature pea seeds contain, besides albumen, two distinct globulins, legumin and vicilin. Danielsson (1952), studying the synthesis of different types of protein in developing pea seeds, concluded that globulins and albumins were synthesized independently, and that the globulins vicilin and legumin were synthesized at different rates, the relative content of vicilin decreasing as ripening proceeded. He noted also that the protein synthesis occurred in detached unripe seeds but ceased at -20°C . Mothes (1939) found that in developing seeds of *L. albus* the ratio of nitrogen to sulphur in the protein remained steady in the earlier stages, but increased sharply in the later stages of ripening. This change implies either the synthesis of sulphur-poor proteins not formed earlier, or a marked increase in the rate of formation of such proteins relative to that of proteins richer in sulphur-containing amino acid residues. A similar trend is apparent in the data of Emmerling (1900) for *V. faba*.

The earlier work on the metabolism of developing seeds emphasized the synthesis of protein, comparatively little attention being given to the changes in carbohydrates. Pfenninger (1909) showed that almost all starch was gone from the hulls of *P. vulgaris* by the end of the ripening period. The loss per hull was only about 25 per cent. of the gain by the seeds of a single pod. Carbohydrate was also thus translocated to the seeds from other parts of the plant, the hull acting as a temporary reservoir. Bisson and Jones (1932) found in the hulls of developing pea pods an early accumulation of starch, which later disappeared almost completely, presumably through translocation to the seeds. Sugar (hexose and sucrose) in the hull showed similar changes with time, but was present in considerably greater amounts than starch. Sugar (almost entirely sucrose) reached an early maximum in the seeds also, and declined later to a steady value. Starch per seed increased throughout development, slowly at first but very rapidly later. Crude fibre, determined as organic material resisting hydrolysis by both dilute acid and dilute alkali, and presumably consisting mainly of cellulose, increased steadily throughout in both seeds and hulls.

(b) Present Studies

The first paper in this series describes for the pea (*Pisum sativum* L.) the changes in the amount per seed and per hull of fresh weight, dry weight, starch, soluble carbohydrate, protein nitrogen, and soluble nitrogen. Results are included for two crops of field-grown peas of the variety Canner's Perfection, representing two seasons and two localities. In each season sampling began when the seeds were very small and continued until they were at or past canning maturity. In one season respiration rates, ascorbic acid, and total and free phosphate were determined at each sampling date, and alcohol-insoluble solids and pectin for the later samples where these determinations could be made on the material available. The soluble nitrogen constituents of seeds and hulls were studied chromatographically in both seasons; these results will be reported in a subsequent paper.

II. MATERIALS AND METHODS

(a) Sampling

Samples of pea pods were taken from crops grown in 1952 at Boree Cabonne, about 20 miles west of Orange, N.S.W., and in 1953 at the Hawkesbury Agricultural College, Richmond, N.S.W. To obtain samples of pods of uniform maturity, blossoms were tagged with coloured wool. The dates of tagging were October 9, 1952 and September 9, 1953. The times between tagging and sampling are shown in Table 3. Each sample consisted of at least 100 pods picked at random. In the 1952 season the pods were separated into seeds and hulls and the total number of each recorded. The material was then placed in cans containing enough ethanol to give a 75 per cent. aqueous solution, the moisture content of the material being taken as 80 per cent. The cans were sealed and mailed to Sydney, together with weighed fresh material for moisture determinations. The pods of the 1953 season were picked in the morning and taken by road direct to the laboratory.

(b) Preliminary Treatment of Samples

In both seasons the average number of seeds per pod and the average weight per seed and per hull were determined as soon as possible after picking. Twenty seeds from each sample were preserved in alcohol-formalin solution for cell size measurements. Fresh material was used for moisture determinations in 1952 and 1953 and also in 1953 for other analyses. In both seasons part of each sample was used for chromatographic investigations, and part was dried for analysis. The alcoholic liquid in which the 1952 samples were preserved was divided by volume and the seeds or hulls by weight. In 1953 the samples were divided by weight and the required amount of alcohol added to each portion.

(c) Drying

The alcohol was evaporated, and the solid material was dried in an air-draught oven at 70°C overnight, and in a vacuum oven at 70°C for 6 hr. The dried material was finally ground and stored in air-tight jars. For moisture determinations, fresh material was weighed into aluminium cans and dried as above.

(d) Respiration Rate

Respiration rate was measured by the Pettenkofer method (Turner 1949) at 25°C for intact pods, seeds alone, and hulls alone, each sample being enclosed in a glass shell. The samples varied according to the expected activity of the material; usually 20 seeds, 9 hulls, and 5 intact pods were taken. Determinations of respiration rate began 3-4 hr after picking, and about 30 min after the separation of seeds and hulls.

(e) Cell Size Measurements

The methods used to measure cell size and to calculate mean cell number per seed, cell volume, and cell surface area were based on those described

earlier for the apple (Bain and Robertson 1951; Robertson and Turner 1951). The testas were removed and the cotyledons cut into halves in a plane parallel to that in which the radicle lay. The sections for cell size measurements were cut from the surface thus exposed. The measurements were made from cells near the middle of the cotyledon section, but mean cell volume did not vary markedly with position in this section. Mean cell volumes were determined for six areas across the section of the cotyledon, using material from the 21-day sample of 1952 and from the 32-day sample of 1953. In both cases the mean cell volume was slightly larger at the ends of the section than in the centre, but the differences were small and of doubtful significance. All cell size values were based on measurements for 50 cells.

TABLE 1
WATER AND SOLIDS IN DEVELOPING SEEDS, 1953

Days after Tagging	Average Number of Seeds per Pod	Fresh Wt. per Seed (mg)	Dry Wt. per Seed (mg)	Water per Seed (mg)	Solids (%)	Alcohol-insoluble Solids (%)
14	5.89	58	10	48	17.0	—
18	5.87	130	24	106	18.4	—
20	6.24	187	34	153	18.2	—
23	5.76	276	55	221	20.0	—
26	5.83	365	84	281	23.1	—
29	6.60	455	100	355	22.0	12.3
32	6.30	466	139	327	29.8	17.9
35	6.00	492	143	349	29.0	22.9
40	6.06	528	185	343	35.0	27.5

(f) Analytical

The nitrogen fractions and starch were determined on dried material as described by Turner (1949). Total soluble carbohydrates were determined by the anthrone method as modified by Johanson (1954), using dried material in 1952 and fresh material in 1953.

Ascorbic acid was estimated on fresh material in the 1953 season by extraction with 3 per cent. metaphosphoric acid and titration with 2,6-dichlorophenolindophenol.

The pectic acid content of the seeds from the later 1953 samples was determined on fresh material using the method described in "Methods of Analysis of the Association of Official Agricultural Chemists"; the alcohol precipitate was weighed directly as pectic acid, without reprecipitation as the sodium salt.

Alcohol-insoluble solids (A.I.S.) were determined by blending fresh material with 80 per cent. alcohol and weighing the filtered residue after drying for 2 hr at 110°C.

Total phosphorus was determined by the method of Allen (1940) using dry material, digested for 30 min with 60 per cent. perchloric acid. Inorganic phosphate was determined after extraction of dry material with 8 per cent. trichloroacetic acid. Ester phosphate was taken as the difference between total acid-soluble phosphorus, estimated after digestion of the trichloroacetic acid extract with perchloric acid, and the inorganic phosphate.

III. RESULTS

Earlier workers have used varying terms for the parts of the pea fruit. In this paper "pod" means the whole fruit, "hull" the carpel wall, and "seed" is used in the botanical sense.

TABLE 2
WATER AND SOLIDS IN DEVELOPING HULLS, 1953

Days after Tagging	Fresh Wt. per Hull (mg)	Dry Wt. per Hull (mg)	Water per Hull (mg)	Solids (%)
14	2110	279	1831	13.2
18	2780	413	2367	14.9
20	3052	466	2586	15.3
23	2702	318	2384	14.8
26	2770	456	2314	16.4
29	3321	457	2864	13.8
32	2282	447	1835	19.6
35	1918	389	1529	20.3
40	1648	321	1327	19.5

(a) Fresh Weight

The seeds contained at the start of sampling in 1952 8 per cent. of the total fresh weight of the pod; in 1953 the proportion was 14 per cent. The fresh weight of the seeds increased consistently over the whole period studied; that of the hull increased consistently until about 20 days after tagging. About 30 days after tagging in 1953, the fresh weight of a hull was approximately equal to that of the seeds contained in it. After about 30 days there was a fall in the fresh weight of the hull, while that of the seeds remained steady or increased less rapidly than before. The fresh weight per seed was consistently higher in the 1952 than in the 1953 season. In 1952 the seed weight at 27 days after tagging was 600 mg; in 1953 the corresponding value (derived by interpolation between the values for 26 and 29 days) was about 400 mg. Similar seasonal differences were observed for most of the other substances estimated in the seeds and hulls. Detailed results for the 1953 season are given in Table 1 for seeds and in Table 2 for hulls. The data on fresh weights, dry weights, and water content in both seasons are summarized in Figures 1 and 2.

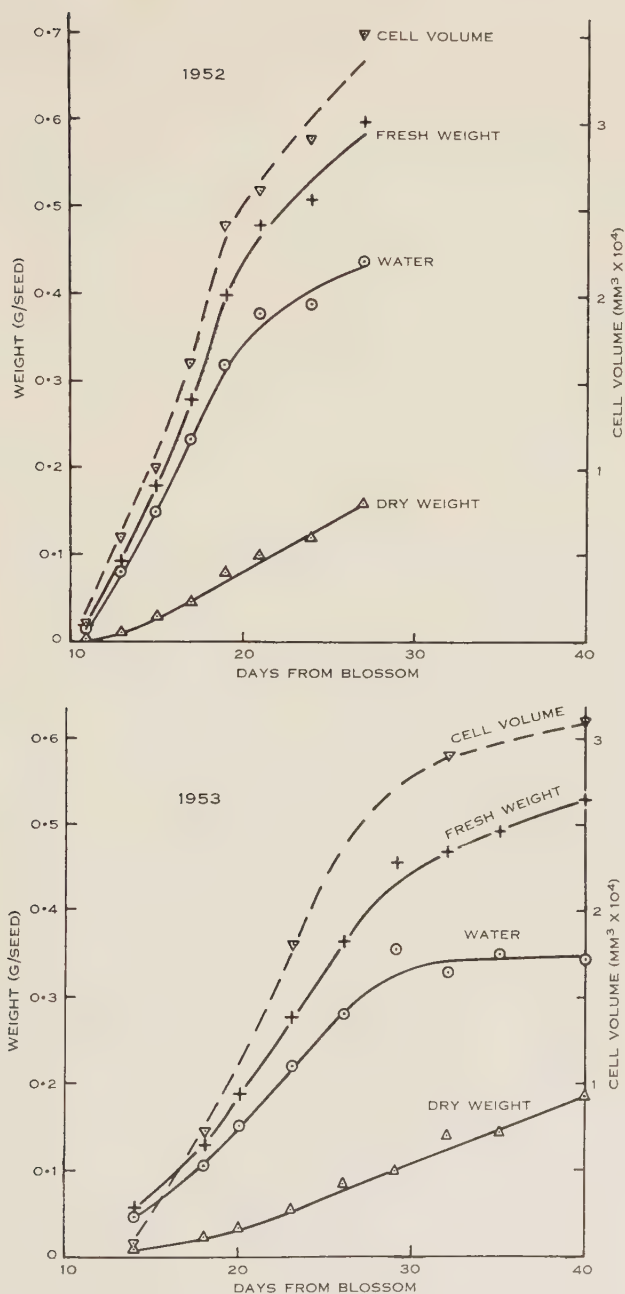


Fig. 1.—Increase in fresh weight, dry weight, water, and mean cell volume in seeds with time from blossom in both seasons.

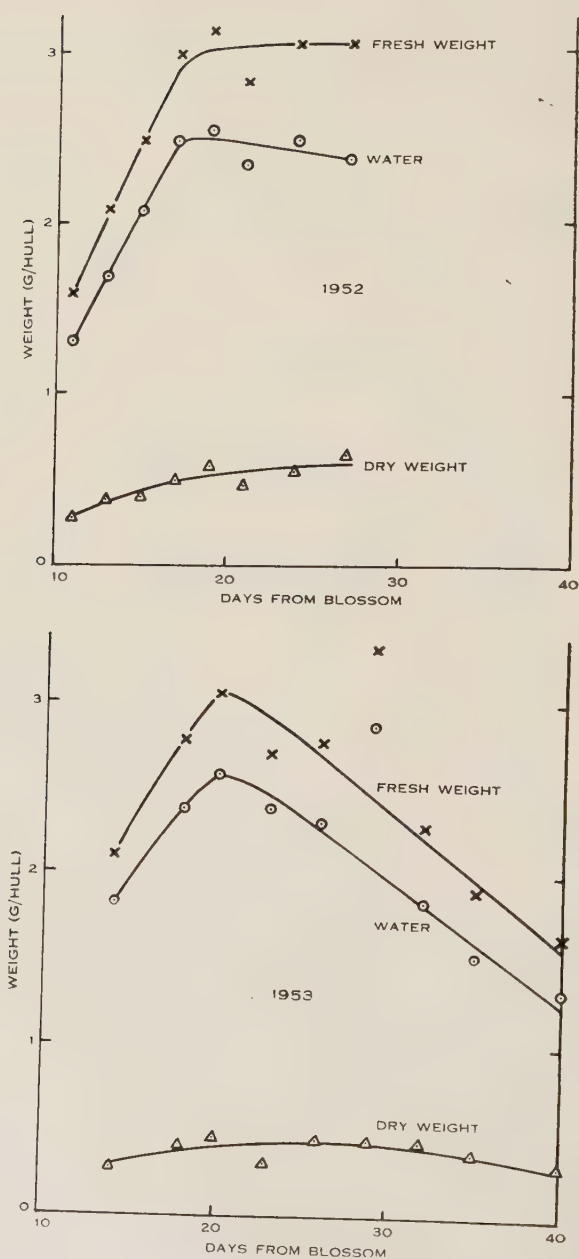


Fig. 2.—Changes in fresh weight, dry weight, and water in hulls with time from blossom in both seasons.

(b) Dry Weight

The dry weight per hull increased in the early part of the period studied. Between 20 and 30 days it appeared essentially steady, except for a temporary decrease at 23 days. The dry weight per seed increased up to the end of the period studied in each season. In the earlier samples the dry weight of the hulls exceeded that of the seeds they contained, but after 26 days there was in each pod more dry matter in the seeds than in the hulls. The changes in dry weight are shown in Figures 1 and 2.

TABLE 3

CHANGES IN CELL VOLUME, CELL SURFACE, AND CELL NUMBER DURING DEVELOPMENT OF PEA SEEDS (COTYLEDONS ONLY)

1952				1953			
Days after Tagging*	Cell Volume (cu. mm. $\times 10^{-4}$)	Cell Surface (sq. mm.)	Cell Number ($\times 10^6$)	Days after Tagging†	Cell Volume (cu. mm. $\times 10^{-4}$)	Cell Surface (sq. mm.)	Cell Number ($\times 10^6$)
11	0.1	0.010	1.3	14	0.08	0.007	0.7
13	0.6	0.031	1.3	18	0.73	0.031	1.4
15	1.0	0.044	1.7	20‡	1.3	0.043	
17	1.6	0.053	1.7	23	1.8	0.059	1.3
19	2.4	0.074	1.5	26‡	2.3	0.068	
21	2.6	0.079	1.6	29‡	2.6	0.075	
24	2.9	0.085	1.6	32	2.9	0.080	1.3
27	3.5	0.095	1.5	35‡	3.0	0.082	
				40	3.1	0.084	1.4

* Tagging date October 9, 1952. † Tagging date September 9, 1953. ‡ Interpolated.

(c) Cell Volume and Cell Number

In both seasons the cell volume increased rapidly from the earliest samples taken (Fig. 1). In 1952 there was a reduction in the rate of increase in cell volume at about 20 days after tagging. In 1953 a distinct reduction in the rate of increase in cell volume occurred after about 24 days. Cell volume in 1952 at the last sample (27 days after tagging) was 3.5×10^{-4} cu. mm., and at the same day in 1953 it was, as estimated by interpolation between the values for 26 and 29 days, 2.4×10^{-4} cu. mm.

Cell number showed little change during most of the period of the observations; apparently cell division was largely completed before the seed reached a weight of 130 mg. In 1952 the estimated cell number per seed was 1.3×10^6 for each of the first two samples, taken at 11 and 13 days. Subsequent samples taken from 15 to 27 days showed minor fluctuations about a mean number of 1.6×10^6 . In 1953 the estimated cell number in the earliest sample, taken at 14 days but apparently less mature than the 11-day sample of the previous year, was 0.7×10^6 . All the subsequent samples, from 18 to 40 days, gave estimated

cell numbers per seed between 1.3×10^6 and 1.4×10^6 . The seeds of the 1953 series had thus both fewer and smaller cells than those of the 1952 series. The data on cell measurements are summarized in Table 3.

(d) *Carbohydrates*

Changes in soluble carbohydrate followed the same general pattern in both seasons, though in each season fluctuations of unknown origin were observed in the soluble carbohydrate content of the hulls. Results for the 1953 season are given in Table 4 for the seeds and Table 5 for the hulls. Changes in both seasons are shown in Figures 3 and 4. In each year both the hull and its seeds contained, for most of the period studied, considerable amounts of soluble carbohydrate. The soluble carbohydrate per seed started at a low level

TABLE 4
CHANGES IN CARBOHYDRATES AND PECTIN DURING DEVELOPMENT OF SEEDS, 1953

Days after Tagging	Soluble Carbohydrate			Starch			Pectin Per Seed (mg)
	Per Seed (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)	Per Seed (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)	
14	—	—	—	0.8	14	81	—
18	—	—	—	2.5	19	103	—
20	14	75	412	3.2	17	93	—
23	21	77	383	7.8	28	142	2.7
26	27	74	321	20	54	234	8.0
29	15	33	148	26	57	258	10.9
32	17	36	120	46	100	334	—
35	12	25	86	52	105	362	14.8
40	17	33	93	74	140	399	21.4

and rose steadily until about 25 days after tagging, when a decline set in. The highest content of soluble carbohydrate in the hulls occurred a few days later. Fluctuations in the soluble carbohydrate content of the hulls and in the seeds were often in opposite directions, so that a much smoother time curve is obtained by plotting soluble carbohydrate per pod than when either the hulls or the contained seeds are considered separately. In the earliest samples there was a moderate amount of starch in the hulls and very little in the seeds. A rapid accumulation of starch in the seeds began about 20 days after tagging in the 1953 series, and a few days earlier in the 1952 series. During the earlier part of this accumulation in the seeds the starch content of the hulls showed no significant change, but it began to decrease between 20 and 25 days after tagging, and soon fell to a negligible amount.

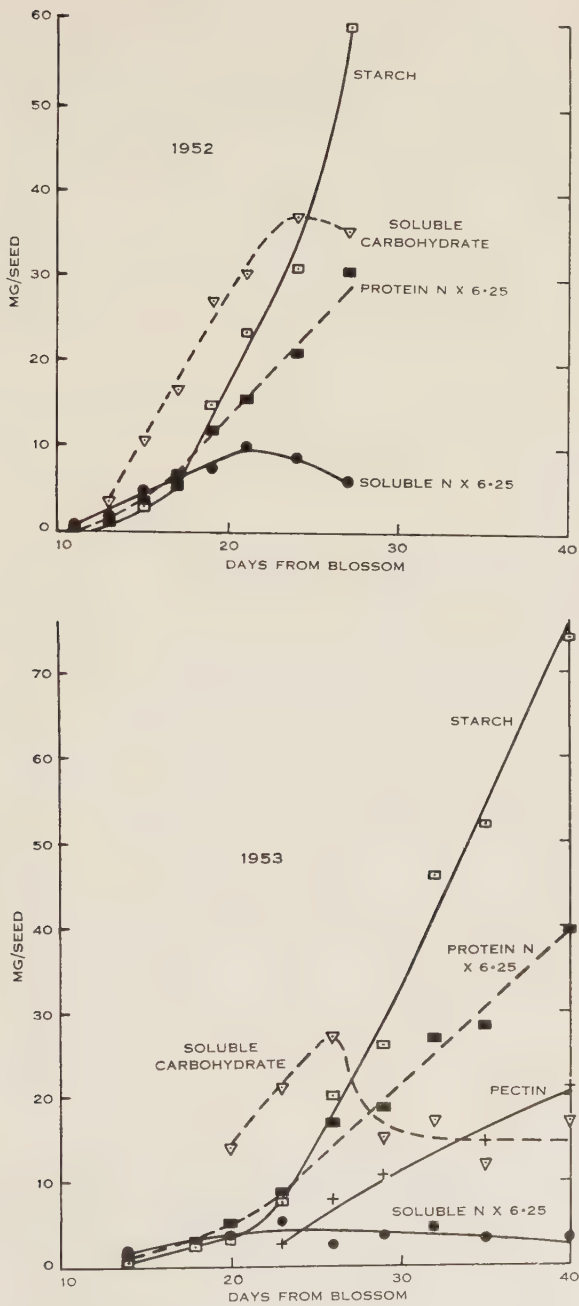


Fig. 3.—Starch, soluble carbohydrate, protein nitrogen, and soluble nitrogen in seeds of both seasons, and pectin in seeds of the 1953 season, plotted against time from blossom.

(e) Nitrogenous Substances

Total nitrogen per seed increased throughout the period studied, the absolute amounts being higher in 1952 than in 1953. Total nitrogen per hull showed marked fluctuations in each season, but no consistent decrease occurred until

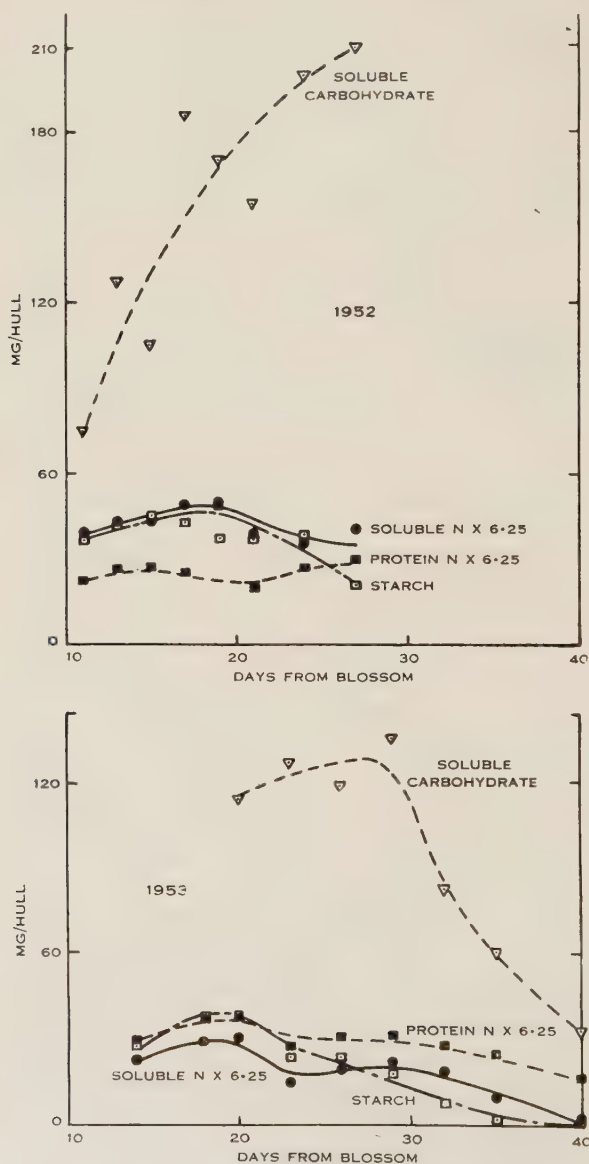


Fig. 4.—Starch, soluble carbohydrate, protein nitrogen, and soluble nitrogen in hulls of both seasons plotted against time from blossom.

30 days after tagging. At about 17 days after tagging in 1952 and 21 days in 1953 total nitrogen content in the seeds of a pod passed that of the hull, and

thereafter greatly exceeded it. The picture for protein nitrogen was very similar to that for total nitrogen, showing a similar steady increase per seed while marked fluctuations occurred in the hulls, with a subsequent decrease late in the period studied. After 20 days the increase in protein nitrogen per seed remained approximately linear, the daily increment being 0.36 mg in 1952 and 0.27 mg in 1953. The maximum content of soluble nitrogen per seed was reached at 21 days after tagging in 1952, and at 23 days in 1953. As with soluble carbohydrate, fluctuations in the hulls and in their contained seeds were usually in opposite directions. Changes in total protein and soluble nitrogen are illustrated in Figures 3 and 4, and data for 1953 given in Tables 6 and 7.

(f) *Ascorbic Acid*

This was estimated on the seeds in 1953 only. The content per seed rose steeply until 28 days after tagging, then showed only minor fluctuations to

TABLE 5
CHANGES IN CARBOHYDRATES DURING DEVELOPMENT OF HULLS, 1953

Days after Tagging	Soluble Carbohydrate			Starch		
	Per Hull (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)	Per Hull (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)
14	—	—	—	28	13	102
18	—	—	—	40	14	97
20	114	37	245	39	13	84
23	128	47	319	24	12	76
26	119	43	263	24	8.7	53
29	136	41	297	18	5.5	40
32	80	35	180	7	2.9	15
35	61	32	157	2	1.0	5
40	33	20	103	1	0.4	2

about 35 days; there was a marked decrease between 35 and 40 days. The ascorbic acid content per unit fresh weight rose slightly from 14 to 20 days, and then fell fairly steadily until 40 days. The data are shown in Figure 5.

(g) *Cell Wall Substances*

Alcohol-insoluble solids (Table 1) and pectin (Table 4; Fig. 3) were estimated only on the seeds from the later samples of the 1953 season. The content per seed of each of these increased very steadily with time. When the values for pectin (Table 4) are compared with those for cell surface (Table 3) it can be seen that pectin increased rapidly while cell surface was increasing only slowly, suggesting thickening of the cell walls by pectin.

(h) Total Phosphorus, Ester Phosphate, and Inorganic Phosphate

Total phosphorus increased steadily in seeds over the whole period from 14 to 40 days; the hulls showed somewhat erratic values, but a marked downward trend was obvious (Fig. 6). Inorganic phosphate, which had been in-

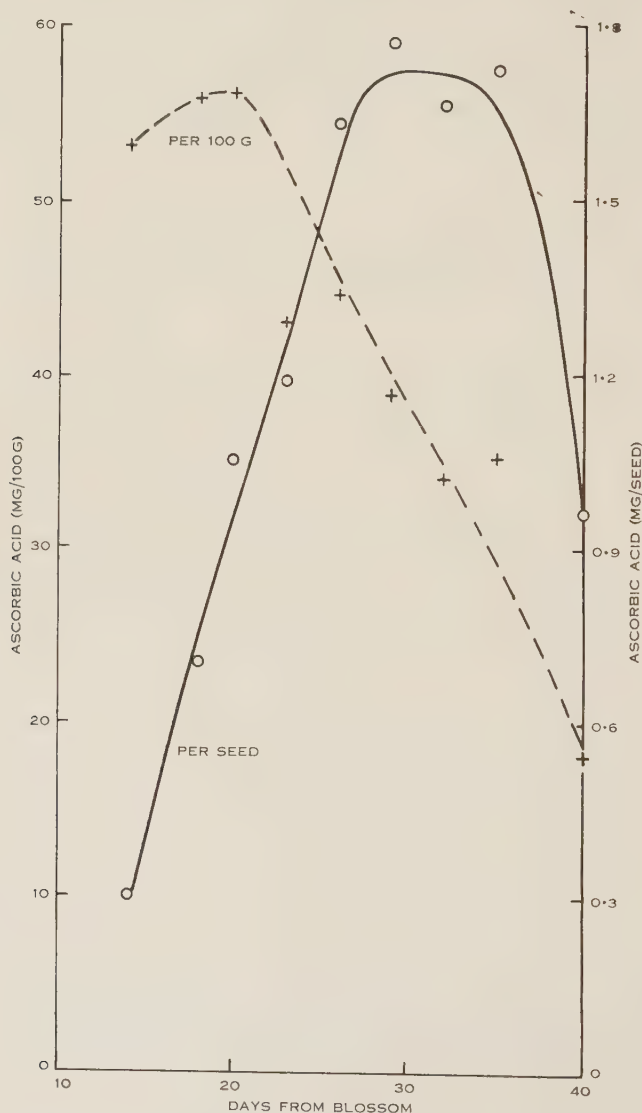


Fig. 5.—Ascorbic acid content of seeds in the 1953 season plotted against time, both as amount per seed and as amount per unit fresh weight.

creasing at a lower rate than the total phosphorus, actually decreased between the 20th and 23rd days. There was a corresponding increase in ester phosphate; this may have an important bearing on the synthesis of carbohydrate.

(i) Respiration

The respiratory output of carbon dioxide (beginning 3-4 hr after picking and 30 min after separation of seeds and hulls) was determined in 1953 only, on intact pods, and on carefully separated seeds and hulls. The data are shown in Figure 7 (unit basis) and Figure 8 (fresh weight basis). From 14 to 35 days after tagging the respiratory rate per unit fresh weight was considerably higher for isolated seeds than for isolated hulls or intact pods. Between 35 and 40 days, a sharp decrease brought the respiration rate of the seeds below those of the hulls or intact pods. The rate for intact pods showed no marked

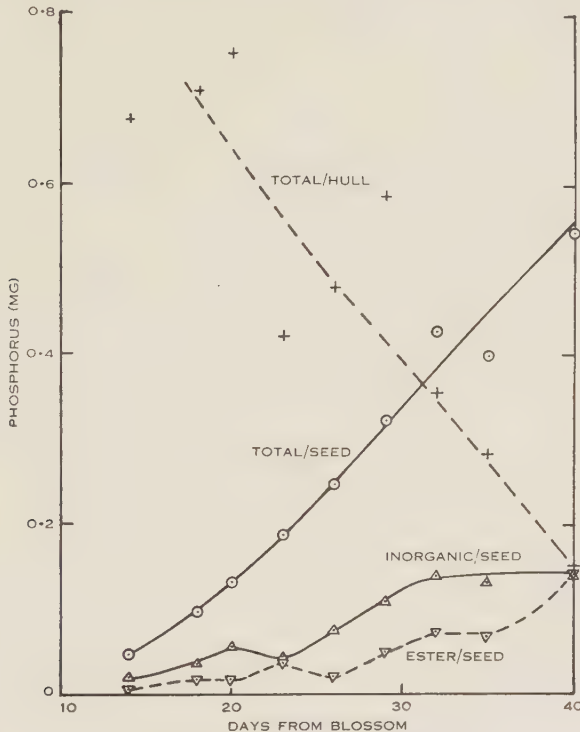


Fig. 6.—Total phosphorus in seeds and hulls and inorganic and ester phosphorus in seeds plotted against time from blossom in the 1953 season.

change over the whole period observed between 14 and 40 days. The rate for intact pods is always less than that to be expected from the rates for the seeds and hulls separately. The seeds in the intact pods thus respire at a lower rate than those removed from it. It is not clear whether this is to be attributed to stimulation due to extra handling of material from opened pods, or to some factor, possibly the accumulation of carbon dioxide or deficiency of oxygen, reducing the respiratory activity of seeds in the intact pods. The latter explanation is supported by the fact that the hulls, which on opening the pods were damaged more than the seeds, nevertheless respired at a much lower rate than the seeds.

IV. DISCUSSION

(a) Seasonal Effects

The seeds developed more rapidly and accumulated larger storage reserves in the 1952 than in the 1953 season. The reasons for this are uncertain. The 1953 crop, grown at Boree Cabonne on the Western Slopes of New South Wales, developed during a period of night frosts; no frosts were encountered by the 1953 crop, grown near Richmond at the western edge of the coastal plain. In each year the developing fruit received adequate moisture, that of 1952 from rainfall only and that of 1953 from rainfall supplemented by irrigation sprays. The pea plants in 1952 showed that concentrated burst of flowering and maturation of fruit which makes the variety (Canner's Perfection) popular as a single-harvest canning crop. In 1953, however, the plants showed extensive second

TABLE 6
CHANGES IN TOTAL, SOLUBLE, AND PROTEIN NITROGEN DURING DEVELOPMENT OF SEEDS, 1953

Days after Tagging	Total Nitrogen			Protein Nitrogen			Soluble Nitrogen		
	Per Seed (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)	Per Seed (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)	Per Seed (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)
14	0.5	8.6	51	0.2	3.9	23	0.3	4.7	28
18	1.0	7.9	43	0.5	3.6	20	0.5	4.3	23
20	1.4	7.2	40	0.8	4.2	23	0.6	3.0	17
23	2.3	8.4	41	1.4	5.2	26	0.9	3.2	15
26	3.1	8.6	37	2.7	7.4	32	0.4	1.2	5.4
29	3.6	8.0	36	3.0	6.9	30	0.6	1.1	5.9
32	5.0	10.6	36	4.3	9.2	31	0.7	1.4	4.7
35	5.0	10.0	35	4.5	9.0	31	0.5	1.0	3.4
40	6.8	12.8	37	6.3	11.8	34	0.5	1.0	2.7

growth, and continued to flower and set fruit even after maturity of the first lot of fruits. That this continued growth was in part at the expense of the pods formed earlier was suggested by the smaller size and content of reserves in the seeds of the 1953 season compared with those of the 1952 season. The crops in the two seasons grew in different weather conditions as well as in different localities. The effect of locality *per se* may not be large, for Kurgatnikov (1936) found that the seeds of 12 varieties of peas all retained their characteristic proportions of starch and protein, which varied considerably between varieties, when grown at each of three widely separated localities with very different climates. In the present work differences in the absolute amounts of the various constituents of the seeds and hulls in each season were not accompanied by any marked divergence in the relations between individual consti-

tments. The discussion is therefore based mainly on the data for the 1953 season, the series of samples being longer and the range of analyses wider than for the 1952 season.

(b) *Distribution of Materials within the Plant*

The general picture of events is similar to that found by earlier workers, especially Bisson and Jones (1932), who studied another variety of pea, Dwarf Telephone. The absolute contents of total solids, total nitrogen, starch, and protein increased in both seeds and hulls in the earliest stages they investigated. Later the continued increase of these constituents in the seeds was associated with their decrease in the hull, which apparently acts as a temporary reservoir

TABLE 7

CHANGES IN TOTAL, SOLUBLE, AND PROTEIN NITROGEN DURING DEVELOPMENT OF HULLS, 1953

Days after Tagging	Total Nitrogen			Protein Nitrogen			Soluble Nitrogen		
	Per Hull (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)	Per Hull (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)	Per Hull (mg)	Per Gram Fresh Wt. (mg)	Per Gram Dry Wt. (mg)
14	8.4	4.0	30	4.7	2.2	17	3.7	1.8	13
18	10.5	3.8	25	5.8	2.1	14	4.7	1.7	11
20	11.0	3.6	24	5.9	1.9	13	5.1	1.7	11
23	6.9	3.2	22	4.4	2.0	14	2.5	1.2	7.9
26	8.4	3.0	18	5.1	1.8	11	3.3	1.2	7.3
29	8.8	2.7	19	5.2	1.6	11	3.6	1.1	7.9
32	7.6	3.3	17	4.5	2.0	10	3.1	1.3	6.9
35	5.8	3.0	15	4.0	2.1	10	1.8	0.9	4.7
40	3.0	1.8	9.5	2.6	1.6	8.3	0.4	0.2	1.2

of minor importance, storing materials later translocated to the seeds. Such a transfer of materials has been observed in detached pods in experiments in this laboratory which will be described in detail later. The amounts lost from the hull are, in fruits attached in the normal way to the plant, considerably smaller than the concurrent increase in the seeds contained by the hull. This point is illustrated in Table 8, which includes for comparison values derived from the data of Bisson and Jones (1932) for the variety Dwarf Telephone. The values in Table 8 show that throughout the period of development the increase of each constituent in the seeds of a single pod exceeds its concurrent decrease in the hull, usually by a factor of several times. The reserves stored in the seeds are therefore mainly derived from materials translocated from outside the pod, even in the later stages of development when there is an appreciable net export from the hull. Little of the starch and protein laid down in the seed can be derived from soluble precursors translocated to it earlier, in spite of the

distinct drop in the later stages in the content per pod of soluble carbohydrate and of soluble nitrogenous compounds. In the 1953 season, starch per pod rose by 173 mg between 26 and 35 days after blossoming; in the same period soluble carbohydrate fell by 141 mg. Assuming that the respiration rate of pods on the plant was not greatly different from those determined 3-4 hr after the pods were picked, the respiratory output of carbon dioxide over this period would be

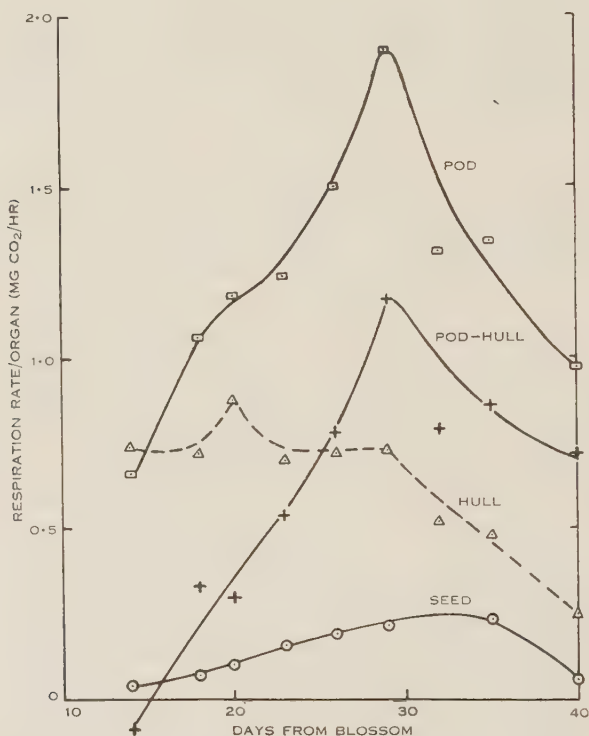


Fig. 7.—Respiration rate per pod, per hull, and per seed plotted against time from blossom. *Pod-Hull*, respiration rate per hull subtracted from that of the intact pod, giving an estimate of the respiration rate of the seeds in the unopened pod.

equivalent to 239 mg of hexose per pod, so that respiration alone accounts for more soluble carbohydrate than disappears, the balance presumably being formed by photosynthesis in the hull or imported from other parts of the plant. From 23 to 40 days after tagging, the soluble nitrogen per pod decreased steadily, but the total loss of soluble nitrogen per pod was 5.7 mg, compared with an increase of 26 mg in the protein nitrogen over the same period.

The relations between various constituents in the hulls and in the seeds can be illustrated in another way as shown in Figure 9, where the percentage in the seeds of the total content of various substances in the whole pod (i.e. hull plus seeds) is plotted against time for the 1953 season; similar curves con-

structed from the data of Bisson and Jones (1932) are included for comparison. Both sets of data show a generally similar picture. The partition of starch, protein nitrogen, total nitrogen, water, and (in the data of Bisson and Jones) ash favoured the seeds to a steadily increasing degree throughout the period studied. The proportion of water and ash finally remaining in the hull was, however, much greater than for starch, protein nitrogen, or total nitrogen. The partition of soluble nitrogen was somewhat erratic; that of soluble carbohydrate was still more erratic, and has been omitted to avoid undue difficulty in reading the graphs.

TABLE 8

INCREMENTS (MG/DAY) IN VARIOUS CONSTITUENTS OF HULL AND SEEDS IN DEVELOPING PEA PODS

Days after Tagging	Present Work (1953 season) Variety Canner's Perfection			Bisson and Jones (1932) Variety Dwarf Telephone		
	14-23	23-29	29-40	12-24	24-32	32-40
Total solids (seeds)	29.0	56.9	41.8	34.7	44.0	34.0
Total solids (hull)	13.4	9.5	-12.3	18.7	-8.0	-20.8
Starch (seeds)	4.5	21.0	25.2	4.6	11.8	11.2
Starch (hull)	-0.5	-1.0	-1.6	0.6	-3.2	-3.0
Total N (seeds)	1.5	1.2	1.5	1.3	1.6	1.1
Total N (hull)	-0.2	-0.2	-0.2	0.1	-0.4	-0.6

No account has been found in the literature of any experiments designed to check whether the pea plant at blossoming contains enough nitrogen for development of the seeds. Studies on other species suggest, however, that an intake of nitrogen from outside the plant is likely at this stage. Such an intake is clearly shown by the data of Emmerling (1900) for the broad bean (*Vicia faba*); Figure 10 summarizes a part of his very extensive data on the distribution of nitrogen between different parts of the plant. He found a large and continuous increase in nitrogen content per plant throughout the period of rapid nitrogen accumulation in the seeds. As the seeds developed, the nitrogen content of the leaves decreased rapidly. Here, as in the pea, the hull is a minor reservoir of nitrogen for the developing seeds. Both in the stem and in the root the absolute content of nitrogen increased steadily throughout the life of the plant, though neither ever contained a large proportion of the total nitrogen except in the earliest stages of growth. A decrease of nitrogen in the leaves of annual plants, concurrent with its accumulation in ripening seeds, has been recorded in maize (Hay, Earley, and de Turk 1953), barley (Deleano and Gotterbarm 1936), and sunflower (Gouwentak 1929, 1931).

The composition of the dry matter at two stages in the development of the seed is shown in Table 9. Starch formed 33 per cent. of the dry matter at 32 days, and 40 per cent. at 40 days. Protein and pectin were also major con-

stituents at both stages. Similar trends appear in the data of Bisson and Jones (1932), though even with the inclusion of ash and crude fibre (presumably cellulose) they accounted for only 64 per cent. of the dry matter at 40 days. In our analyses unknowns amounted to about 20 per cent. at 32 days and 10 per cent. at 40 days. Ash, judging from the data of Bisson and Jones, would account for about 4 per cent. Further work may define the unknown constituents; cellulose may play an important part in the toughening of the maturing seeds.

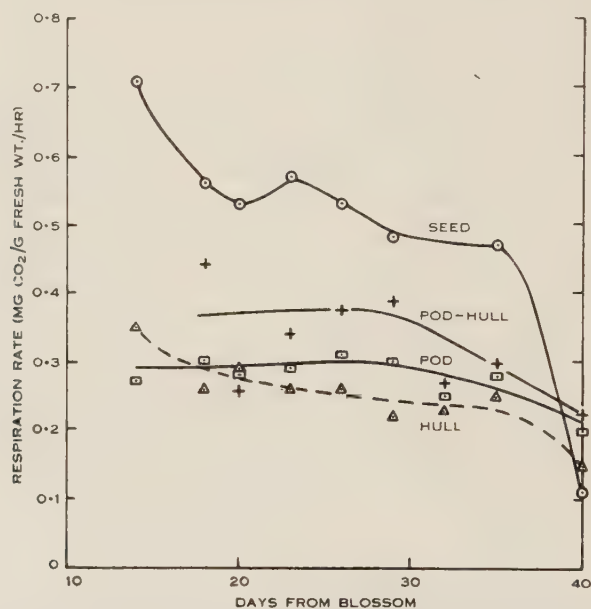


Fig. 8.—Respiration rates per unit fresh weight in pods, hulls, and seeds. The rate for the seeds in the intact pod (Fig. 7) is also included.

(c) Carbohydrate Synthesis

Two spectacular changes in carbohydrate balance occurred in the developing pea seeds. A marked increase in the rate of starch synthesis occurred at about 17 days in 1952 and at about 21-22 days in 1953. Soluble carbohydrate dropped sharply at about 24 days in 1952 and at about 26 days in 1953 (Fig. 3). Similar changes appear in the results of Bisson and Jones. These sharp changes pose interesting problems concerning the control of synthetic processes. Soluble carbohydrate (shown by Bisson and Jones to be mainly sucrose) accumulated before the rapid increase in starch, suggesting that carbohydrate reaching the seed as sucrose was transformed to starch.

Turner (1953) demonstrated that extracts from pea seeds can synthesize sucrose from a mixture of glucose-1-phosphate and fructose. This synthesis was not due to a simple reaction between glucose-1-phosphate and fructose. In the developing pea seed sucrose is probably being broken down. If the synthetic mechanism were working in reverse, each sucrose molecule could thus

form one molecule of glucose-1-phosphate, capable of direct condensation to starch, and one molecule of fructose, whose transformation to starch or use in respiration would require preliminary phosphorylation. Such processes seem consistent with the decrease in free phosphate per seed between 20 and 23 days (representing a large decrease in concentration, as cell volume is increasing rapidly at this stage). A corresponding increase in ester phosphate was

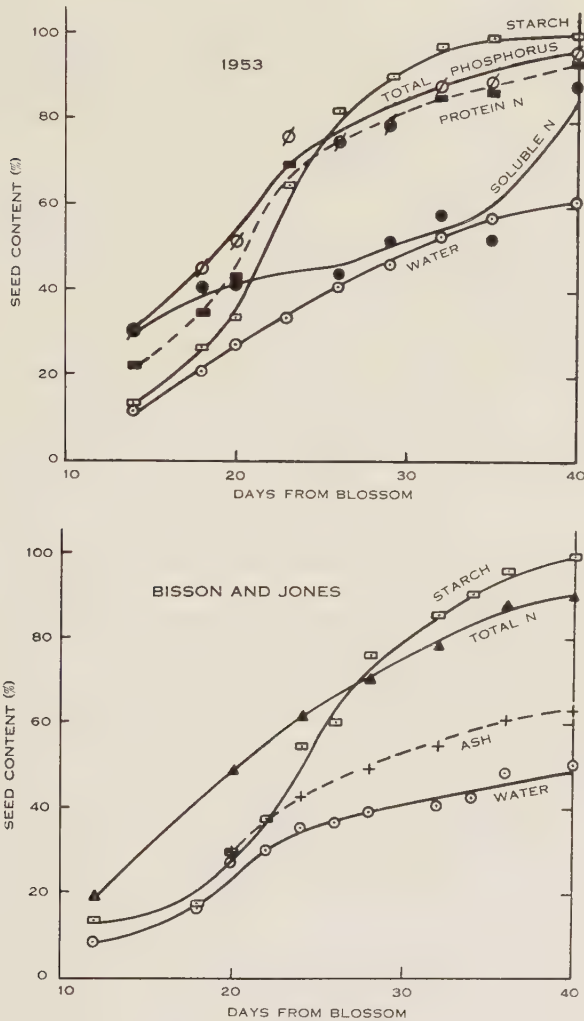


Fig. 9.—Occurrence of various substances in the seeds expressed as a percentage of the total amount of each substance in the whole pod, plotted against time from blossom. Similar curves constructed from the data of Bisson and Jones are shown for comparison.

followed by a decrease during the early stages of the active starch synthesis. It is significant that in another variety of pea an accumulation of hexose mono-phosphate before the main period of starch synthesis has been demonstrated

by Rowan (personal communication) in investigations forming part of the present programme of research. Results of this work will be published in a later paper of this series. The mechanism controlling the transition from accumulation of hexose monophosphate to their condensation to starch remains unknown. One possibility is that these syntheses depend on the formation in the cells of the appropriate enzymes, a process recalling the enzymatic adaptations known in microorganisms.

(d) *Soluble Nitrogenous Compounds and Protein Synthesis*

The problems of controlling mechanism raised by the changing relations between protein and soluble nitrogenous compounds resemble those for carbohydrates. The rate of protein synthesis rose about the same time as that of starch synthesis. This increase in the rate of protein synthesis coincided with a decrease in soluble nitrogenous compounds, which entered the seed at a lower rate than they were used in protein synthesis. Little is known at present about the form in which nitrogen is translocated.

TABLE 9
BALANCE SHEET FOR SEEDS, 1953

Constituent	32 Days after Tagging		40 Days after Tagging	
	Weight (mg)	Per Cent. of Dry Wt.	Weight (mg)	Per Cent. of Dry Wt.
Dry weight	139	100	185	100
Soluble carbohydrate	17	12.2	17	9.2
Starch	46	33.2	74	40.0
Protein nitrogen $\times 6.25$	27	19.4	39	21.1
Soluble nitrogen $\times 6.25$	4	2.9	3	1.7
Ascorbic acid	2	1.4	1	0.5
Pectin	13	9.4	21	11.4
A.I.S. minus (starch + protein + pectin)	-2	-1.4	11	6.0
Phosphate	1	0.7	2	1.1
Total	108	77.8	168	91

The pea seed is an organ storing protein, and its cells are in sharp metabolic contrast with those of the parenchyma of apple fruits (Robertson and Turner 1951), where protein content is correlated with cell surface, implying the synthesis of cytoplasmic proteins only. Evidence is cited in Section I for the accumulation of specific storage proteins in the cotyledons of the developing pea seeds.

(e) *Pectin in Seeds*

Pectin, like starch and protein, took part in the intense synthetic activity beginning, in the 1953 season, at about 21 days. At 23 days the pectin content was still low, but thereafter it increased continuously. It can be seen in Tables 3 and 4 that pectin increased relatively more rapidly than cell surface between 23 and 40 days, suggesting that it was used in thickening cell walls as well as in increasing their area. Such an increase in the thickness of cell walls might be associated with the toughening of the seed during this period. It seems probable that synthesis of protein and of pectin, as well as that of starch, depends on phosphorylated compounds.

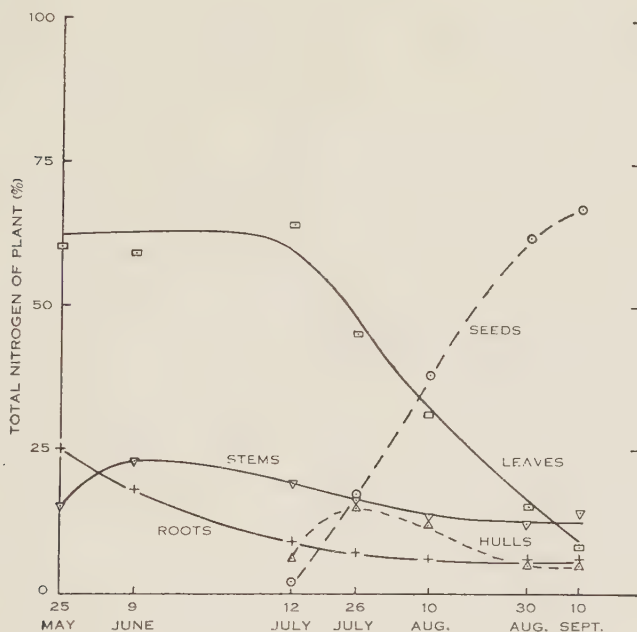


Fig. 10.—Distribution of nitrogen between different parts of the plant of *Vicia faba* from the data of Emmerling. The nitrogen in each organ is expressed as a percentage of the total nitrogen in the plant at each time.

(f) *Respiration and Synthesis*

The possibility that phosphorylated compounds are involved in the intense synthesis leading to the simultaneous rapid production of starch, protein, and pectin emphasizes the need for information about the respiratory process, the final source of energy used in synthesis. It is also important to know whether the seed receives phosphorylated intermediates, or itself performs the phosphorylations. The difficulty of assessing the true respiration rate of the seeds in their natural conditions within the hull makes caution necessary in comparing the available data for respiratory and synthetic processes.

The respiration of the seeds, whether measured by the output of carbon dioxide per unit fresh weight or per seed, fell off markedly between 35 and 40 days after tagging. This is at first sight somewhat surprising in view of the substantial formation of protein, starch, and pectin in the seeds during this period. It is possible, however, that these syntheses do not require a large

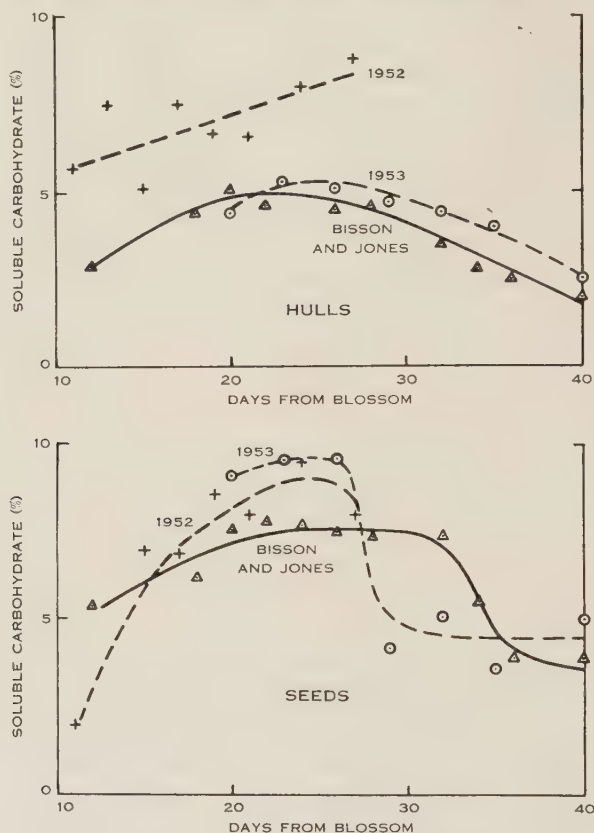


Fig. 11.—Changes in the soluble carbohydrate concentration (soluble carbohydrate per seed or hull as a percentage of the water in the seed or hull) with time in both hulls and seeds of both seasons. Similar curves from the data of Bisson and Jones are included.

supply of energy direct from respiration at this stage. Starch and protein, for instance, might be formed from hexose phosphate previously formed and amino acids or phosphorylated intermediates of protein synthesis requiring a comparatively small transfer of phosphate, so that no correlation need necessarily be expected between changes in the rate of synthesis and that of respiration. The drop in respiration rate between 35 and 40 days appears not to be due to shortage of substrate. Over this period there is no appreciable decrease in the content of soluble carbohydrate per seed, as was found also for total sugars by Bisson and Jones (1932). It may be important to note that the lowered respiration rate at this time was accompanied by an increase in unknown phos-

phorylated compounds, because free phosphate remained unchanged in amount while total phosphate increased (Fig. 6). Phytate, which is known to occur in pea seeds, may account for some of this phosphate.

(g) *Water Content*

Of the substances estimated, water per seed shows a break in the time curve which might be correlated with the sharply defined change in quality which marks the harvesting point giving a maximal yield of first class canning peas. Protein, starch, and particularly pectin might also be expected to be concerned in physical changes in the seed, but the contents of all these rise smoothly over the relevant period.

In the 1953 season the water content per seed rose almost linearly up to 29 days, after which there was no consistent change up to 40 days, when the last sample was taken. This cessation of water accumulation by the seed was without effect on its increase in dry matter, which was approximately linear between 20 and 40 days. There was, however, a sharp fall between 26 and 29 days in the concentration of soluble carbohydrates in the cell sap of the seeds. The changes in this concentration (calculated as the soluble carbohydrate per seed expressed as a percentage of the water per seed) are shown in Figure 11. The 1953 values, and those calculated from the data of Bisson and Jones (1932), both show this sudden fall in concentration about this time judged to be optimal for harvesting, accompanied by an actual decrease in water content in the data of Bisson and Jones. A similar break a few days earlier in the concentration of total soluble nitrogenous compounds was well shown in the data for 1953, and appeared in the 1952 data also, though here only one sample was taken after the break. No similar data are available from the work of Bisson and Jones (1932), who estimated only total nitrogen. It should be noted that in the 1953 season, though water uptake ceased, the seeds continued to increase in cell size and the increasing dry matter caused an increase in fresh weight.

(h) *Problems being Investigated*

This preliminary survey suggests many further problems. A study of the soluble nitrogen compounds in seeds and hulls is nearing completion. Work on the changes in phosphates and phosphorylated compounds, including the phosphate carriers adenosine diphosphate and adenosine triphosphate, during development has begun and will be related to other changes, particularly those in respiration rate and carbohydrate content. A survey of the changes in individual soluble carbohydrate constituents will begin shortly. Some work has also been done on post-harvest changes to determine whether similar trends persist after picking.

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STUDIES IN THE METABOLISM OF PLANT CELLS

X. RESPIRATORY ACTIVITY AND IONIC RELATIONS OF PLANT MITOCHONDRIA

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Summary

The respiratory activity and ionic balance of mitochondria isolated from carrot and beet tissues by differential centrifugation have been studied. The oxygen uptake of the mitochondria with different substrates was investigated. The mitochondria hold both cations and anions in concentrations greater than those in the supernatant. Experiments on the time of adjustment to a changed concentration of chloride in the supernatant solution have been used to calculate the diffusion constant of salt in the particle. On the assumption that most of the resistance to diffusion is in the surface membrane (thickness about 200 Å), the apparent diffusion constant of chloride in the membrane was shown to be of the order of 10^{-13} cm²/sec. This agrees with that found for heart muscle sarcosomes under similar conditions and is of the order expected in a lipo-protein membrane. The concentrations of mobile cations (Na⁺ and K⁺) in the mitochondria are considerably greater than those in the supernatant. It is suggested that the internal concentrations are largely due to a Donnan equilibrium based on the immobile anions of the particle. Since no simple Donnan equilibrium will account for the simultaneous concentration of both mobile cations and mobile anions, it is suggested that the mobile anions might be accumulated by an accumulatory mechanism. The anion concentration difference between the inside and outside of the particles is of the order of magnitude to be expected if the electron carrier of respiration were acting as the anion carrier of accumulation. The results are therefore not inconsistent with the earlier hypotheses for the interdependence of the two processes. The results support the hypothesis that mitochondria are probably involved in electrolyte accumulation in plant cells and in secretion in animal cells such as those of the gastric mucosa.

I. INTRODUCTION

The participation of a cytochrome oxidase system in the salt respiration of cut plant tissue has been demonstrated (Weeks and Robertson 1950). It has been suggested that salt accumulation depends on salt respiration (Lundegårdh 1940; Robertson and Turner 1944) and that the mechanism might be associated with the cytochrome system. Robertson and Wilkins (1948) showed that the quantitative relation between salt respiration and accumulation is consistent with the hypothesis that the separation of hydrogen ions and electrons at the cytochrome system is part of the mechanism.

If the cytochrome system is part of the mechanism of salt accumulation it is desirable to know where this system of enzymes is located in the cell. By

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analogy with animal enzymes it was suggested (Robertson 1951) that since the cytochrome probably occurred on the mitochondrial particles, the mitochondria are implicated in the processes of salt respiration and accumulation. Since then Stafford (1951) and Millerd (1951) have established that plant cytochrome oxidase occurs in intracellular particles, of the size of mitochondria. Further, Millerd *et al.* (1951) showed that, as with animal cells, other respiratory enzymes occur on these particles. These results have been confirmed by Davies (1953), Laties (1953*a*, 1953*b*), and Brummond and Burris (1954).

The purpose of this paper is to record investigations on the respiratory activity and ionic relations of intracellular particles from carrot and beet tissue. This work was done in conjunction with a study of the morphology of the particles by phase contrast and electron microscopy (Farrant, Robertson, and Wilkins 1953, and unpublished data). The study of the ionic relations of the plant cell particles is essential to the consideration of their possible role in accumulation. Accumulation of cations in animal mitochondria has been demonstrated by Bartley and Davies (1952, 1954), Macfarlane and Spencer (1953), and Stanbury and Mudge (1953); Bartley and Davies also demonstrated that phosphate and organic acid anions were concentrated in mitochondria.

II. MATERIAL

The xylem parenchyma of carrot root (*Daucus carota* L.) and the heterogeneous tissues of the beet root (*Beta vulgaris* L.) were used. These tissues were chosen because they have been used for respiration and accumulation studies in earlier work. Prior to blending, the tissue was prepared in the cold by cutting into cubes of sides approximately 0.5 cm. Cytochrome *c* was prepared by the method of Keilin and Hartree as quoted by Umbreit, Burris, and Stauffer (1949). The final dialysis was made against distilled water instead of sodium chloride solution.

III. METHODS

The particles were extracted in the cold by disintegrating the tissue, separating the larger constituents by squeezing through muslin, and centrifuging at different speeds to separate the particles. As the tissues consist largely of mature, highly vacuolated cells, a large volume of tissue was necessary to obtain a relatively small number of mitochondria. Facilities for preparing the particles were improved as the work progressed.

(a) Preparation of Particles

The tissue was disintegrated in a Waring Blendor for 30 sec. Approximately equal volumes of tissue and blending medium were used in the blendor bowl. Sucrose (15 per cent.) was found to be the most satisfactory blending medium as judged by oxygen uptake. In both beet and carrot tissues the homogenate came to a pH of approximately 6; neutralizing the medium with potassium hydroxide during blending did not improve the activity.

After blending, the preparation was squeezed through two layers of muslin. Most nuclei and larger pieces of cell debris were satisfactorily separated from

the homogenate by centrifuging for 5 min at 500 *g*. In early experiments the mitochondria were separated by centrifuging for 57 min at 1800 *g* or at forces greater than 10,000 *g* for a few minutes. When facilities for intermediate speeds became available centrifuging for 25 to 30 min at 5000 *g* was adopted as a standard procedure.

In some experiments the mitochondrial fraction was "washed" by resuspending in 50 ml of the blending medium and centrifuging for 40 min at 1800 *g*. A centrifuging time of 15 to 20 min at 5000 *g* was used for particles prepared at 5000 *g*. The endogenous oxygen uptake of mitochondria washed once was considerably lower than that of unwashed particles but was rarely reduced to zero. It was unnecessary to wash mitochondria even once to detect additional activity when some of the substrates were added.

For respiratory studies, the particles were resuspended in a small volume of a solution of the same composition as that in which the tissues had been blended. For ionic balance studies, the washed particles were resuspended directly in the medium in which they were to be studied. The preparation of the particles up to and including the stage of resuspension (for respiratory studies), and up to the stage of resuspension (for ionic studies) was carried out in the cold, in most experiments between 0 and 5°C.

(b) Measurement of Respiratory Activity

Oxygen uptake was measured by standard Warburg technique. The temperature of the thermostat was either 25 or 27°C. The temperature chosen was below that at which maximum activity could be obtained.

In all experiments all constituents of the medium were placed in the main compartment of the Warburg vessel. A total of 4 ml including 1 ml particle suspension, which was added last, was used in each vessel. After various trials to determine conditions for maximum activity it became standard practice to add cytochrome *c* (approx. 1.6×10^{-5} M in vessel), phosphate buffer pH 7.0-7.4 (0.025 M in vessel), and sucrose (15 per cent. in vessel) to all vessels, and substrate acids, neutralized with sodium or potassium hydroxide before adding, to some vessels. This mixture is conveniently referred to as "medium." Adenosine triphosphate and magnesium sulphate were added as required but not used in all experiments.

Oxygen uptake is expressed in several ways. For comparison within an experiment $\mu\text{l O}_2/\text{ml suspension/hr}$ is a convenient expression. The best method of expression of results for comparison both within and between experiments is as $\mu\text{l O}_2/\text{mg nitrogen/hr}$. Nitrogen was estimated by the Nessler method after digestion of 0.5 ml mitochondrial suspension.

(c) Ion Balance

Investigation of the ion balance required that the mitochondrial particles be resuspended in the solutions in which they were to be investigated, aerated for various periods at 25°C, and then centrifuged and analysed for changes in ion concentration. The ions investigated were potassium, sodium, and chloride.

In some experiments the prepared particles were resuspended directly in the medium which was to be investigated. In others, the prepared particles were resuspended and "washed" by centrifuging before being resuspended in the medium to be investigated. The "washing" medium was varied according to requirements.

Particles were washed in six centrifuge tubes. The pellet in each tube was weighed and held at 0°C till required. The pellet in one tube was resuspended immediately in 5 or 3.7 ml of its own supernatant and centrifuged at once for 2 min at 25,000 *g* at 25°C. The pellet thus obtained was used to determine the concentration of ions in the particles initially. The pellets in the remaining tubes were each resuspended in 5 or 3.7 ml 15 per cent. sucrose (for loss experiments) or in medium containing substrate + KCl (for uptake experiments). At least one replicate was held in a Warburg vessel for measurement of oxygen uptake; in some experiments all were held in Warburg vessels to measure oxygen uptake. The remaining tubes were held, shaking for the required period at 25°C, and then centrifuged at once for 2 min at 25,000 *g* at 25°C. The suspension from the Warburg vessels was also centrifuged for 2 min at 25,000 *g* at 25°C.

Immediately after centrifuging, the supernatant was poured off and kept for analysis. The pellet was treated in the following way: (i) The tube was drained and the sides blotted with filter paper. (ii) the pellet was weighed and the particles resuspended in 6 or 4.5 ml water. (iii) A sample was removed for nitrogen estimation. (iv) The suspension was transferred to a test tube, weighed, heated in a boiling bath for 30 min to disorganize the particles, and weighed again. (v) This suspension was filtered, and the filtrate was then ready for chloride, potassium, and sodium estimations.

Chloride was determined by the electrometric titration method described by Best (1929), adapted for small samples, 0.5-1.0 ml, by use of an "Agla" micrometer syringe for the titration. The sample was acidified with nitric acid to suppress ionization of organic acids before titration.

Sodium and potassium were determined with a flame photometer as described by Domingo and Klyne (1949). The filtrate to be tested was diluted with 0.006M NaCl for K determinations and 0.006M KCl for Na determinations, and compared with standards of K in 0.006M NaCl and of Na in 0.006M KCl to overcome the interference of the two ions (Termer, Eggleston, and Krebs 1950). The accuracy of these determinations was ± 5 per cent.

Concentrations thus determined represent the concentration of ions in the mitochondrial pellet and, if the mitochondria accumulate the ions, represent an underestimate of the concentration within the particles themselves because an unknown amount of the supernatant solution will be packed between the particles. If the particles remain spherical, the amount of solution packed between them, with optimum packing, would be about 30 per cent. of the pellet volume and with less efficient packing may be 50 per cent. of the pellet volume. Added to this would be an unknown volume of liquid in the meniscus above the pellet. Bartley and Davies (1954) estimated the percentage of extra-

particulate water in their mitochondrial preparations as 66 per cent. Independent measurements (Hope, unpublished data) suggest that the extra-particulate water in our preparations was not more than 50 per cent.

IV. RESULTS

(a) Respiratory Activity

(i) *Enzyme Activity of Different Fractions.*—Rapid oxygen uptake was obtained in cell-free extracts from beet and carrot tissue, with the substrates succinate, malate, aconitate, and pyruvate. Citrate was also tested but did not

TABLE 1
COMPARISON OF FRACTIONS FROM BEET TISSUE

Tissue (400 g) blended in 15% sucrose (400 ml), squeezed through muslin. Homogenate (590 ml) centrifuged 5 min at 240 g giving 1st supernatant and 1st residue. Centrifuged 1st supernatant 1 hr at 1800g to give 2nd supernatant and 2nd residue. Portion of 2nd residue resuspended in 15% sucrose and centrifuged 40 min at 1800g to give 2nd residue, washed

Fraction	No Substrate	0.08M Sodium Succinate	0.08M Sodium Malate
Oxygen Uptake ($\mu\text{l O}_2/\text{g fresh wt./hr}$)			
Whole disks	120-140	117	117
Oxygen Uptake ($\mu\text{l O}_2/\text{ml fraction/hr}$)			
Homogenate	11	20	18
1st Supernatant	15	21	18
1st Residue	28	226	174
2nd Supernatant	8	11	10
2nd Residue	200	1680	1260
2nd Residue, washed	119	1180	716

increase the oxygen uptake. As has been shown with other plant tissues (Miller *et al.* 1951) the activity of the enzymes which oxidize these substrates is associated with particles of approximately 1μ diameter. This is clearly shown when various fractions are compared on the basis of activity per ml fraction (Table 1). No attempt was made to ensure complete recovery of the particles.

(ii) *Oxygen Uptake with Different Substrates.*—The oxygen uptake with different substrates was qualitatively the same with beet and carrot mito-

chondria. Results showing the oxygen uptake in the presence of different substrates are given in Tables 1 and 2 for beet and in Table 3 for carrot. The oxygen uptake with succinate was consistently high. In most experiments on beet tissue the oxygen uptake in the presence of malate was approximately 70

TABLE 2
OXYGEN UPTAKE BY BEET MITOCHONDRIA IN DIFFERENT SUBSTRATES

Centrifuging: 5 min at 500 g, 20-30 min at 5000 g

Experiment	Oxygen Uptake ($\mu\text{l O}_2/\text{ml/hr}$)			
	No Substrate	0.08M Sodium Succinate	0.08 Sodium Malate	0.08 Sodium Pyruvate
1	32.4	196	—	40
2	36	249	181	—
3	48	410	294	107
4	74	447	332	53
5	76	708	342	69
6	14.5	186	76*	—

* 0.04M Sodium malate.

per cent. of that in succinate while in carrot tissue it was only about 50 per cent. Increase of oxygen uptake with pyruvate was always very small but over the series of experiments the rate with pyruvate was greater than that with no

TABLE 3
OXYGEN UPTAKE BY CARROT MITOCHONDRIA IN DIFFERENT SUBSTRATES

Centrifuging: 5 min at 250 g, 40 min at 1600 g, 15 min (wash) at 1800 g. Substrate concentration: 0.04M

Added Factors	Oxygen Uptake ($\mu\text{l O}_2/\text{ml/hr}$)			
	No Substrate	Succinate	Malate	Aconitate
—	3	46	16	19
Phosphate, pH 6.7	9	47	26	18
Phosphate, pH 6.7 +Mg (0.001M)				
+ATP (0.0005M)	13	55	31	14

added substrate. Aconitate increased the oxygen uptake of carrot mitochondria; beet mitochondria were not tested with aconitate. Citrate did not increase but sometimes slightly depressed the oxygen uptake in both carrot and beet mitochondria.

(iii) *Oxygen Uptake under Different Conditions.*—(1) *Cytochrome c.* The presence of cytochrome *c* was not essential for an active oxygen uptake by mitochondria either with or without substrate. However, the oxygen uptake was enhanced by a small amount of cytochrome *c* as shown in Table 4.

TABLE 4

EFFECT OF CYTOCHROME *c* CONCENTRATION ON OXYGEN UPTAKE BY CARROT MITOCHONDRIA AT 27°C AND BY BEET MITOCHONDRIA AT 25°C

Blending medium: 15% sucrose. Expt. 1, 2, and 3: carrot mitochondria; centrifuging: 2 min and 40 min at 1150 *g*; substrate concentration 0.05M. Expt. 4: beet mitochondria; centrifuging: 5 min at 500 *g*, 30 min at 5000 *g*; substrate concentration 0.08M

Experiment	Concentration of Cytochrome <i>c</i> in Vessel (M × 10 ⁻⁵)	Oxygen Uptake (μl O ₂ /ml/hr)	
		Sodium Succinate	Sodium Malate
1	0	23	—
	0.6	31	—
	1.3	30	—
	1.9	31	—
2	0	17	—
	1.0	32	—
	2.0	34	—
	2.9	33	—
3	0	21	—
	2.5	28	—
	3.8	24	—
4	0	406	144
	0.2	446	174
	0.4	494	206
	0.9	510	210
	1.8	522	220

(2) *Phosphate.* As with cytochrome *c*, the presence of phosphate was not essential for an active oxygen uptake by mitochondria but usually increased the uptake of oxygen with succinate as substrate (Table 5) and always with malate as substrate (Table 1). Experiments with increasing concentrations of phosphate showed that a low concentration (0.01M) was more than sufficient to give a maximum effect (Table 5).

(3) *Magnesium sulphate and adenosine triphosphate.* In several experiments these substances were added to the mitochondria in various combinations with phosphate, no added substrate, and pyruvate as substrate. Effects on oxygen uptake were small but variable, sometimes increasing and sometimes decreasing the rate. They were therefore not added as standard practice (but see also the next paragraph).

(4) *Catalytic Amounts of Malate.* Low concentrations of malate were tested in the presence of pyruvate as substrate (Table 6). Combination of substrate, malate in low concentration, and co-factors increased the oxygen

TABLE 5

OXYGEN UPTAKE OF BEET MITOCHONDRIA IN DIFFERENT CONCENTRATIONS OF PHOSPHATE WITH 0.06M SUCCINATE AS SUBSTRATE

Blending medium: water; centrifuging: 20 min at 1000 g

Preparation	Phosphate Concentration	Oxygen Uptake* ($\mu\text{l O}_2/\text{ml/hr}$)
1	0.024	65.8
	0.018	57.9
	0.012	57.4
	0.006	63.6
	0.001	54.4
	—	36.7
2	0.024	102.9
	0.018	86.6
	0.012	84.1
	0.006	97.7
	0.001	77.8
	—	68.2

*Mean of 2 readings.

uptake above the rates in no substrate and substrate alone. This may be a valuable combination for stabilizing these mitochondria and is being investigated further.

TABLE 6

OXYGEN UPTAKE ($\mu\text{LO}_2/\text{ML}/\text{HR}$) BY BEET MITOCHONDRIA WITH 0.017M PYRUVATE AS SUBSTRATE

Centrifuging: 5 min at 500 g, 20 min at 5000g; concentrations: malate 0.0001M, ATP 0.0005M, Mg 0.001M

No Substrate	Pyruvate	Malate	Pyruvate + Malate	ATP + Mg	Pyruvate + ATP + Mg	Malate + ATP + Mg	Pyruvate + Malate + ATP + Mg
73.8	60.8	65.4	71.4	97.2	97.2	93.6	111.0

(5) *pH.* An experiment in 0.025M phosphate showed no effect of pH over the range 6.8 to 7.6 on oxygen uptake in the absence of substrate and with pyruvate as substrate.

(b) Ion Balance

(i) *Sodium, Potassium, and Chloride in Mitochondria*.—Mitochondria which are extracted from cells have necessarily been exposed to a mixture containing electrolytes which are liberated from the cells into the blending medium. Since these electrolytes have come from both cytoplasm and vacuole, and have been diluted by the sucrose, this supernatant is different from that of the cytoplasm in which the mitochondria occur in intact cells. Since, as will be shown later, mitochondria adjust themselves relatively rapidly to their external solution, no measure of the ionic composition of mitochondria as they exist in intact cells has been obtained.

TABLE 7

CONCENTRATIONS OF SODIUM, POTASSIUM, AND CHLORIDE IN BEET MITOCHONDRIA IN MM/L AFTER EQUILIBRATION WITH THE EXTERNAL CONCENTRATIONS

The number of observations at each concentration is given in brackets. Means and standard errors have been calculated except when there are less than 4 observations

Na ⁺ External Concentration	Na ⁺ Pellet Concentration	K ⁺ External Concentration	K ⁺ Pellet Concentration	Cl ⁻ External Concentration	Cl ⁻ Pellet Concentration
0.6	22.8±2.6 (12)	0.6	11.0±0.8 (17)	0.2	4.9±0.6 (11)
7.13	39.6±3.9 (16)	7.13	20.6±1.9 (5)	5.12	11.7±0.7 (11)
		14.20	21.3±1.6 (5)	13.20	16.9±0.6 (18)
		22.5	28.7		
		23.4	23.8		
		22.8	26.1		
31.2	32.4	32.8	33.2		
33.5	35.6				
30.2	43.4				
		38.6	47.8	45.47	38.4, 39.0
		39.0	50.5		
		44.1	51.3		
		42.7	52.9		
		48.4	58.0		
		49.7	59.2		
79.85	88.1±2.6 (4)				
98.8	79.5				

(ii) *Equilibrium between Mitochondria and External Solution*.—The concentrations of ions released during blending varied with material from different sources, but analyses showed that the concentrations of sodium, potassium, and chloride ions in the mitochondria were greater than the respective concentrations in the supernatants. In a series of experiments the concentrations of sodium, potassium, and chloride were varied in the external medium after preparation and the mitochondria were allowed to adjust themselves to these concentrations before analysis. The concentrations in pellets and supernatants at the time of preparation and in pellets and external media

after adjustment are shown in Table 7. Since it was not always possible to control the external concentrations, the results for external concentrations have been grouped, the corresponding internal concentrations have been averaged, and the standard errors of the means calculated (except when there were less than 4 observations). The mean internal concentrations are plotted against the mid values in the external concentration ranges in Figures 1 and 2. At lower concentrations, the ratios of internal to external concentra-

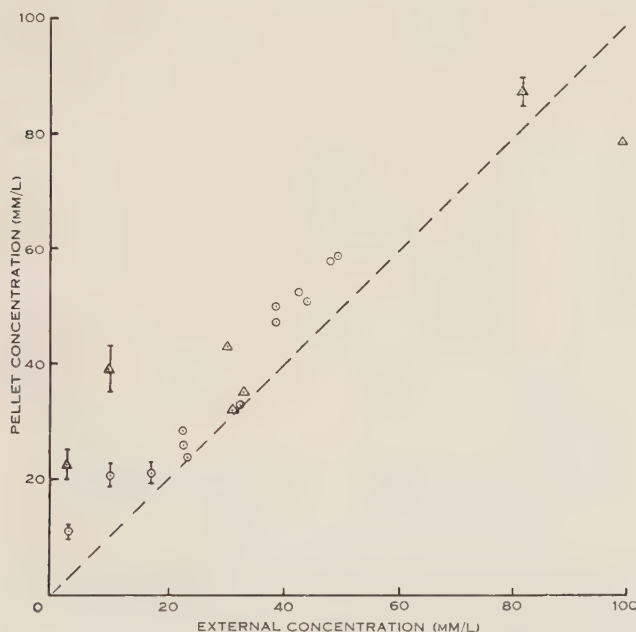


Fig. 1.—Concentrations of sodium and potassium in the pellet plotted against the concentrations in the external solutions. The dotted line shows equality of concentration. Δ , sodium; O, potassium.

tions are high for cations but approach unity at higher concentrations. The ratios of internal chloride to external concentrations are also high at low external concentrations but decrease and become less than unity for external concentrations greater than 18 mM/l. High ratios of internal to external concentrations for either mobile cations or anions would be expected if the adjustment were due to a Donnan equilibrium but, as will be discussed later, a high internal concentration of both mobile cations and anions cannot be explained as a simple Donnan effect. The internal concentration of sodium ions was higher than that of potassium and much higher than that of chloride.

(iii) *Time for Equilibration between Solution and Particles, and Diffusivity of Chloride in the Particle.*—Change in concentration of chloride when the mitochondria were transferred to higher or lower concentrations of chloride

was used to follow the form of the equilibration curve. Figure 3 shows equilibration curves for the transfer to a solution without chloride. Those for transfer to more concentrated solutions (are not plotted here but) have a similar equilibration period.

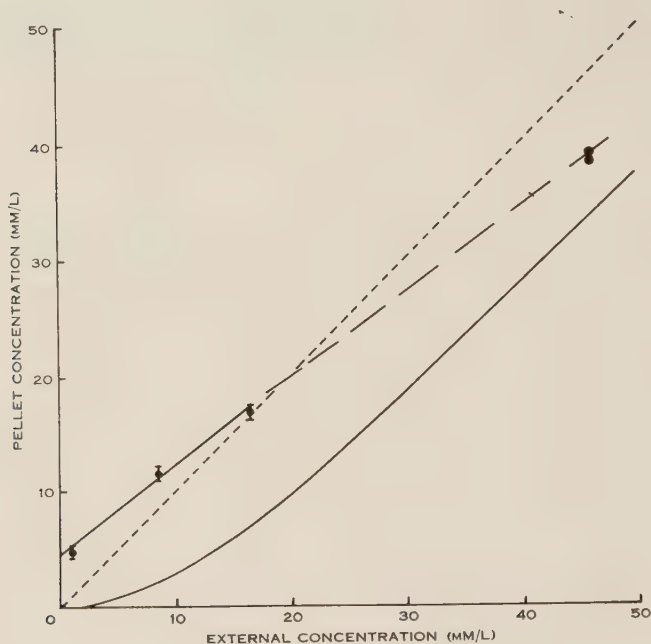


Fig. 2.—Concentration of chloride in the pellet plotted against the concentration in the external solution. The dotted line shows equality of concentration. The curve calculated from the Donnan equilibrium, assuming a concentration of immobile anions (A) of 30 mM/l, is also shown.

(iv) *Ionic Balance in the Mitochondria in Relation to Oxygen Uptake.*—The data given in Table 7 and Figures 1 and 2 show that the concentrations of cations and anions in the mitochondria exceed the concentrations in the external solution. A simple Donnan equilibrium will not explain the concentration of both positive and negative ions, but may be responsible for holding either the cations or the anions in the particles. One possible explanation would be that the proteins in the particles were behaving as anions and holding the cations; at ordinary pH values it seems unlikely that the proteins would be behaving as immobile cations holding the mobile anions.

The oxygen uptake of the pellet might be related to the internal concentration of ions in two ways; firstly, many intermediate compounds formed in metabolism are anionic and would therefore be expected to be balanced by cations, and secondly, one ion may be taken in by an accumulation mechanism dependent on the oxygen uptake. The difference between internal and external

concentrations after the steady state had been attained was measured simultaneously with the oxygen uptake of the particles. The oxygen uptake was examined in a Warburg vessel and the particles were then centrifuged (4 to 7 min) at

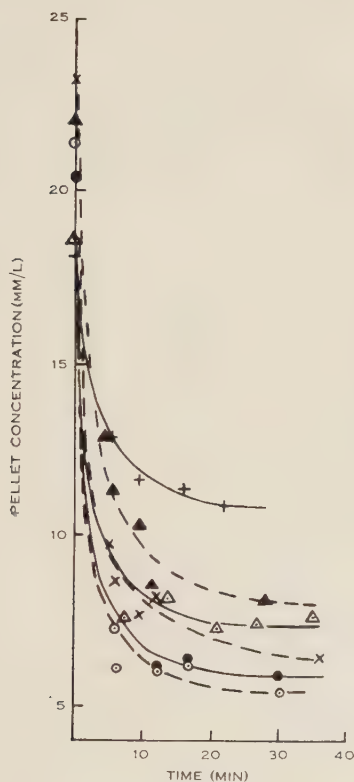


Fig. 3

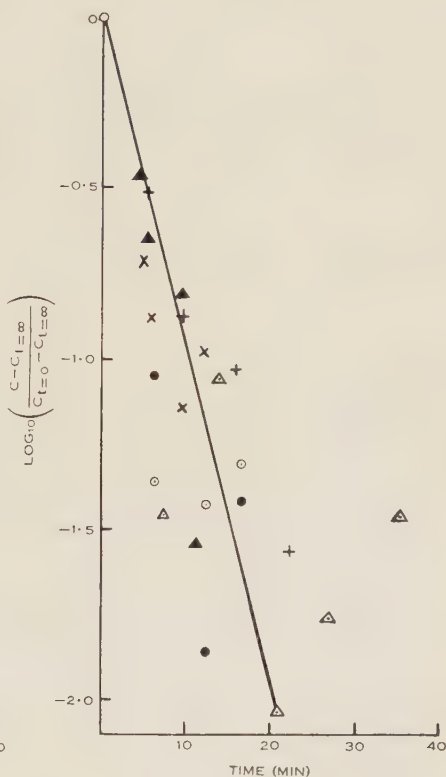


Fig. 4

Fig. 3.—The time-course of adjustment of pellet chloride concentration after change to a lower concentration in the external solution. Different symbols represent different experiments.

Fig. 4.—The data given in Figure 3 have been used to calculate $\log_{10} \left(\frac{c - c_{t=\infty}}{c_{t=0} - c_{t=\infty}} \right)$ (where c is concentration at time t , $c_{t=0}$ is the initial concentration, and $c_{t=\infty}$ is the final concentration), which has been plotted against time.

superspeed at about 25°C. The pellet thus obtained was analysed in the usual way for chloride, sodium, and potassium. The results are shown in Table S and Figure 5. Since the activity of different preparations can be compared only if the results are expressed as the amount of nitrogen which the pellet contains, the results are given as oxygen uptake/mg N and as concentration difference between pellet and supernatant/mg N in the pellet.

V. DISCUSSION

(a) Oxygen Uptake of Extracted Mitochondria

The results confirm the observations of other workers that plant mitochondria can utilize acids of the Krebs organic acid cycle, bringing about in-

TABLE 8

COMPARISONS OF THE DIFFERENCE BETWEEN CONCENTRATIONS OF IONS IN THE MITOCHONDRIA AND SUPERNATANT WITH THE RATE OF OXYGEN UPTAKE

Experiment	Conditions	Oxygen Uptake ($\mu\text{l O}_2$ / mg N/hr)	Differences between Internal and External Concentration					
			Na ⁺ (mM/l)	Na ⁺ (mM/mg N) ($\times 10^6$)	K ⁺ (mM/l)	K ⁺ (mM/mg N) ($\times 10^6$)	Cl ⁻ (mM/l)	Cl ⁻ (mM/mg N) ($\times 10^6$)
RJ 3	0.02M Malate, 0.010M KCl	80.4	55.5	43.3	11.5	9.0	6.4	5.0
	No substrate, 0.019M KCl	73	43.1	73.1	15.1	25.6	2.5	4.2
RJ 4	0.005M Malate, 0.014M KCl	129	29.2	22.3	10.4	7.9	3.7	2.8
	0.005M Malate, 0.014M KCl	108	30.1	22.9	10.7	9.9	1.3	1.2
	No substrate, 0.014M KCl	71.5	38.3	30.1	9.6	7.1	7.1	5.6
	No substrate, 0.014M KCl	39.5	30.8	27.5	8.3	7.4	1.9	1.7
HB 1	0.005M Malate, 0.014M KCl	66			6.2	3.6	4.2	2.4
		54.5			0.4	0.1	4.5	1.5
		75.4			3.3	1.3	1.6	0.6
	No substrate, 0.014M KCl	13.6			1.3	0.7	—	—
		25			1.5	0.6	1.2	0.5
HB 3	0.005M Malate, 0.014M KCl	29.9			1.3	0.7	0.9	0.5
		30.2	32.2	15.3	3.9	1.9	1.7	0.8
		51.3	11.3	7.6	6.6	4.4	6.4	4.3
	No substrate, 0.014M KCl	53	20.0	13.9	7.0	4.9	2.0	1.4
		11.6	5.3	3.8	4.0	2.9	0.2	0.2
		21.9	12.0	6.9	4.1	2.4	0.5	0.3
		10.2	12.0	7.2	6.1	3.6	1.5	0.9

creased oxygen uptake. The utilization of malic and succinic acids by the isolated mitochondria of carrot and beet suggests that the findings of Bennet-Clark and Bexon (1943) and of Turner and Hanley (1949) on tissue slices can be explained by the penetration of the substrates to the mitochondria of the whole tissue where they are utilized as substrates.

Mitochondria from different plant sources after extraction appear to vary widely in their activity as measured by oxygen uptake when different substrate

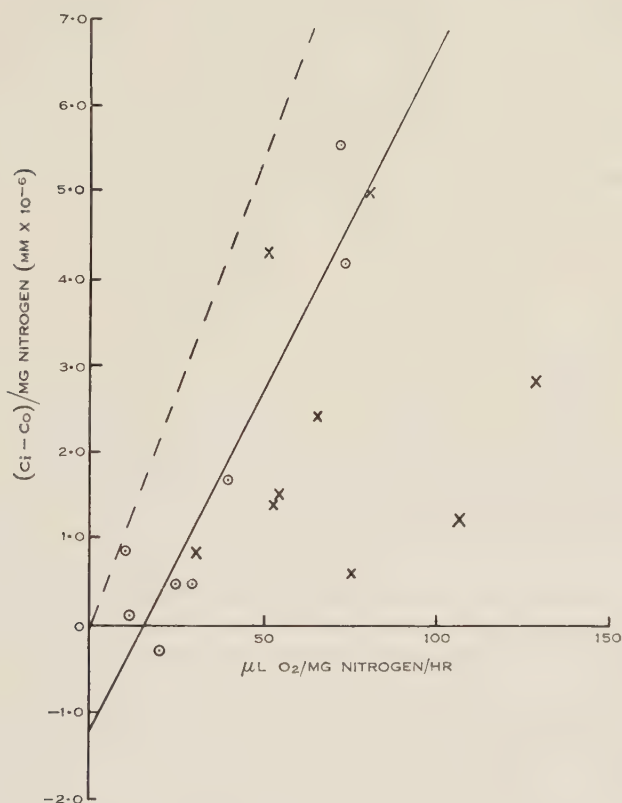


Fig. 5.—The chloride concentration difference between particles and external solution ($c_i - c_o$) plotted against the oxygen uptake. Both are expressed per mg N in the particles. \times , with substrate; \circ , no substrate. The regression line calculated from the no substrate points is shown. The dotted line represents the expected relationship calculated from the hypothesis.

acids are added (Millerd 1952; Davies 1953; Laties 1953*b*). Increase in single step oxidation seems probable in our experiments in which the oxygen uptake with succinate as substrate is greater than that with any other substrate.

(b) Physical Organization of Mitochondria

The experiments on the adjustment to a new concentration of chloride establishes the fact that the mitochondrion offers a resistance to diffusion which makes the apparent diffusion constant very much less than that in water. If the

mitochondrion were treated as a homogeneous sphere the diffusivity of chloride in the body of the mitochondrion would be about 2×10^{-11} cm²/sec which is much less than the diffusivity of chloride in water (1.67×10^{-5} cm²/sec at 20°C). Electron microscope studies, however, have shown that the plant mitochondrion, like the animal mitochondrion, is certainly not a homogeneous sphere (Palade 1952; Farrant, Robertson, and Wilkins 1953) but there is good evidence for an interfacial membrane of the order of 100-200 Å in thickness.

If it is assumed that the mitochondria are spheres approximately 1.0μ in diameter on the average, surrounded by a membrane about 200 Å in thickness (Farrant, Robertson, and Wilkins 1953) the apparent diffusion constant of the chloride in the membrane can be calculated from the equation

$$\log_{10} \frac{c - c_{t=0}}{c_{t=\infty} - c_{t=0}} = - \frac{3Dt}{2.303rl}, \quad \dots \quad (1)$$

where $c_{t=0}$ is the internal concentration at zero time, c is the concentration at time t (in sec), $c_{t=\infty}$ is the equilibrium concentration of the particle, l is the thickness of the membrane (cm), and r is the radius of the particle (cm).

The best way of evaluating D is to plot the left-hand side of equation (1) against time (Fig. 4), measure the slope of the line of best fit, and solve the equation for D . From the results plotted, D is 1.2×10^{-13} cm²/sec. As discussed earlier, however, the pellet concentration is an underestimate of the mitochondrial concentration because of the inter-particulate fluid. If the pellet contained 50 per cent. of inter-particulate fluid, the diffusion constant would be reduced to 0.8×10^{-13} cm²/sec, and if it contained 70 per cent. of fluid, D would be reduced still further. Independent measurements suggest that the volume of inter-particulate fluid is about 50 per cent. It will therefore be adequate to take the diffusion constant of the mitochondrial membrane as being 1×10^{-13} cm²/sec. This apparent diffusion constant can be used to apply Fick's law to calculate leakage rates from mitochondria when the concentration inside is higher than that outside. In calculating the diffusion constant it has been assumed that the morphological membrane (see Farrant, Robertson, and Wilkins 1954) is the main source of the resistance to diffusion of electrolytes in solution. If it were assumed that the resistance was uniformly distributed through the particle, the equation for diffusion in the homogeneous sphere could be used as an alternative and the principal conclusions would not be changed.

If there were no membrane present and diffusion were taking place into or out of a sphere of unstirred solution of similar dimensions to the mitochondrion, it can be shown that equilibration will be virtually complete in a millisecond. As seen from Figure 3, the equilibration time is of the order of minutes. Thus the layer of unstirred water which may surround the membrane of each mitochondrion has a negligible effect on the equilibration time compared with that of the membrane, and further, unless the diffusion constant inside the mitochondrion is very much less than in an aqueous solution, it is reasonable to assume that most of the resistance to diffusion is in the surface membrane. On

the assumption that most of the resistance to diffusion is located in this membrane, which is likely on the basis of the observed swelling properties of these mitochondria in dilute solutions and in water, the calculated diffusion constant of the chloride in this membrane is about 1×10^{-13} cm²/sec. This approaches the order of the diffusion constant suggested by Danielli (1942) for electrolytes in a membrane of orientated lipoids stabilized by protein. A lipo-protein membrane has been suggested by Sjöstrand and Rhodin (1953) for the membrane of the mitochondria of mouse kidney epithelium. Further, impedance measurements on suspensions of beet mitochondria (Hope, unpublished data) suggest that the particles have a membrane of high impedance.

Although, as has been pointed out by Danielli (1942), Fick's law may not be applicable to very thin membranes where the resistance to diffusion is not necessarily proportional to the length of the diffusion path or thickness of the membrane, we have used the apparent diffusion constant for comparison with diffusion across other membranes of similar thickness. The permeability can be calculated from these values by dividing the diffusion constant by the thickness of the membrane.

The determination of the diffusion constant for chlorides in the mitochondrial membrane may point to several important conclusions, for example:

(i) It suggests that free ions inside the mitochondria as prepared are likely to diffuse from the mitochondria into the supernatant in about 10 min after extraction. We do not know how this diffusivity, determined after extraction, is related to diffusivity for the membranes in the unruptured cell. If, however, it is of the same order of magnitude, the mitochondrion probably adjusts itself fairly rapidly to its surrounding cytoplasmic medium. Soluble co-factors, if their diffusion constants were similar, would be lost from the particles on extraction, even though the integrity of the particles is largely preserved.

(ii) The low diffusion constant of chloride helps to explain the osmotic properties of the particles, which are apparently freely permeable to water. Presumably some of the molecules which have been used for maintaining the external concentration (e.g. sucrose) penetrate the membrane less rapidly than the chloride and thus, for a time, maintain the external osmotic pressure, preventing rapid water uptake by the particles.

The only experiments to establish similar permeability data for mitochondria seem to have been those of Cleland (1952) working with heart muscle sarcosomes. Using the reciprocal of the time (in minutes) for a 5 per cent. change in the density of the suspension which corresponded to a known tonicity difference, as a measure of the permeability, Cleland showed that the sarcosomes had the same permeability to potassium chloride, potassium succinate, potassium fumarate, potassium malate, and H_2PO_4^- ions but the permeability to NaCl was about two-thirds of this and to sucrose about one-seventh. From data supplied by Cleland (personal communication), the half times of equilibration of these sarcosomes prepared without "Versene," and undergoing no oxidative phosphorylation, give a membrane diffusion constant of 0.8×10^{-13} cm²/sec, assuming that the barrier to diffusion is mostly in the membrane. With "Versene," the calculated diffusion constant decreases to 1×10^{-14} cm²/sec,

and with "Versene" and oxidative phosphorylation it decreases still further to the order of 2×10^{-15} cm²/sec. So far no effects of "Versene" on the diffusion constant of chloride in plant particles have been found. The difference between sarcosomes and plant mitochondria means that the sarcosomes when undergoing phosphorylation (as they are in the intact cell) would probably require several hours to equilibrate to a change in their surroundings. We do not know whether there is a real difference between sarcosomes, which in the electron microscope show considerable internal structure, and plant mitochondria, which show little, or whether the difference is due to the extraction conditions leading to a greater disorganization of the plant mitochondria. This point requires further investigation, particularly as Chappell and Perry (1954) have shown recently that the swelling in hypotonic solutions of mitochondria from pigeon breast muscle can be reversed by the addition of ATP. These results together with those of Macfarlane and Spencer (1953) indicate the need for a full investigation to determine whether the control of swelling depends on phosphorylations influencing structure, or influencing ion uptake by the particles, or both.

(c) Mechanism of Maintaining Internal Concentrations of Ions

When both positive and negative mobile ions of a salt are held in higher concentration in one phase than another, no simple Donnan equilibrium can explain the distribution, as was shown by Briggs and Petrie (1928) in a discussion of ion accumulation by the whole tissue. Bartley and Davies (1954) have shown that, as in our results, mobile anions and cations in the mitochondria of sheep kidney, exceed the concentration of the external solution. There are three possible explanations of such a situation: (i) the mobile ion of one charge is bound and removed from the aqueous phase inside the cell or particle while the mobile ion of the other charge is held by the immobile ions of opposite charge; (ii) there are two phases inside the cell or particle, one containing immobile anions balanced by the mobile cations, and the other containing immobile cations balanced by the mobile anions but the two phases must be quite distinct; (iii) one ion could be held by a simple Donnan equilibrium while the other ion is maintained by some accumulating mechanism against the concentration gradient.

There is insufficient evidence to choose definitely between these three explanations. Stanbury and Mudge (1953) found that potassium could be retained by liver mitochondria against a concentration gradient and that it was only slowly exchanged under their experimental conditions. However, as Bartley and Davies (1954) have shown, the major portions of the ions held under conditions of active metabolism by the particles are easily exchangeable and we are dealing here with actively metabolizing particles. If compounds which would remove either the cations or the anions from solution exist, they must be destroyed upon the disintegration of the particle. While the second explanation is not excluded, there is at present no evidence in its favour. Plant mitochondria do not seem to be characterized by the transverse internal partitions seen in animal mitochondria (Sjöstrand and Rhodin 1953).

The electrophoretic mobility (McLean, unpublished data) of the plant mitochondria prepared in this Laboratory suggests that the particle has a large surface excess of immobile negative charges. It seems that the third possibility, i.e. that the cation is held by the Donnan effect while the anion is held by an accumulatory mechanism working against the leakage merits further investigation. The concentration of immobile anions due to proteins, phospho-lipids, and other substances such as intermediates of metabolism would balance the excess concentration of sodium and potassium which has been observed inside. This concentration of cations tends to be higher with higher respiration rates (Table 8). It is not clear at present why the concentration of Na^+ should be so much higher than that of K^+ . Similar higher concentrations of cations were observed by Bartley and Davies (1954) and were shown to depend on the metabolic activity of the particles.

The full analysis of the relation between internal and external concentrations of cations is impossible without knowledge of the concentration of immobile anions and of the competing power of sodium and potassium for these anions. An estimate of the probable concentration of the immobile anion (A) can be obtained from the apparent intercepts of the internal sodium and potassium concentrations at zero external concentration, allowing for the cations paired with the chloride at zero. This value is approximately 0.03M. If the Donnan equilibrium were the only factor determining the concentration of the ions, the expected internal chloride concentration, $[\text{Cl}_i]$, neglecting other mobile anions, can be calculated from the equation

$$[\text{Cl}_i] = \frac{1}{2}[(A^2 + 4c_0^2)^{1/2} - A], \quad \dots \quad (2)$$

where c_0 is the external concentration of chloride. The expected internal concentrations of chloride are shown in Figure 2, where it can be seen that the internal concentrations of chloride at lower external concentrations are considerably in excess of those expected from a simple Donnan equilibrium. At higher concentrations the internal chloride could be consistent with the Donnan effect being mainly responsible.

Some other mechanism must therefore be suggested to explain the accumulation of mobile anions. The anion concentration difference is not as great as that of the cation. From the diffusivity of Cl^- already determined, the leakage rate if the particle were free in solution in the mitochondria can be calculated from the equation

$$-\frac{dc}{dt} = \frac{3D}{rl}(c_i - c_0), \quad \dots \quad (3)$$

where $(c_i - c_0)$ is the concentration difference between inside and outside (from our experiments about $3 \text{ mM/l} = 3 \times 10^{-6} \text{ g-mol/c.c.}$), D is the diffusivity of Cl^- in the membrane ($1 \times 10^{-13} \text{ cm}^2/\text{sec}$), r is the radius of the particle ($0.5 \times 10^{-4} \text{ cm}$), and l is the thickness of the membrane ($2 \times 10^{-6} \text{ cm}$). The rate of leakage from the mitochondria would be then $9 \times 10^{-9} \text{ g-mol/c.c./sec}$. Since each pellet of mitochondrial suspension weighs about 0.14 g, i.e. is approximately 0.14 ml, the leakage rate/pellet would be $4.5 \times 10^{-6} \text{ g-mol/pellet/hr}$. If now this concentration difference is maintained over some time, in the steady

state, this leakage rate must be balanced by an equal accumulation rate, i.e. 4.5×10^{-6} g-mol Cl^- /pellet/hr.

As mentioned in the introduction, the hypothesis that the accumulation mechanism depends on the transfer of an anion into the cell when an electron passes through the electron carrier system has been shown to be consistent with the quantitative data for accumulation rates and respiration rates in whole tissue (Robertson and Wilkins 1948). It seemed worth investigating whether the accumulation rate, shown above to be necessary for the observed concentration difference between mitochondria and surrounding solution, would have the right quantitative relation to the rate of oxygen uptake. The hypothesis that the accumulation mechanism depends on the transfer of an anion by an electron carrier requires that the maximum accumulation rate should approximate to four times the oxygen uptake in g-mol (since 4 electrons are required in the reduction of one oxygen molecule to water). Thus an accumulation rate of 4.5×10^{-6} g-mol/pellet/hr would require an oxygen uptake of 1.1×10^{-6} g-mol O_2 /pellet/hr or $27.5 \mu\text{l}$ O_2 /pellet/hr. Since the average pellet weighs about 0.14 g and contains about 1.4 mg nitrogen, the expected relationship between the concentration difference $(c_i - c_o)$ /mg N in the pellet and the oxygen uptake/mg N/hr can be calculated and is shown in Figure 5. This figure also shows the difference between internal and external chloride concentrations of the experiments in Table 8. When no substrate is added, the correlation of the difference between internal and external concentration with the oxygen uptake is high ($r = 0.913$, $P < 0.01$); the regression line is shown in Figure 5. When substrate is added the correlation with oxygen uptake is low ($r = 0.15$, $P > 0.5$), and therefore not significant. Thus the difference between internal and external concentrations is of the order of magnitude which would be expected if the accumulation of chloride by the hypothetical mechanism were off-setting the leakage, and the correlation with oxygen uptake is high. Theoretically it would be better to examine the relation of the difference between internal chloride concentration and that calculated from the Donnan equilibrium, to the oxygen uptake; uncertainty about the correction for interparticulate fluid and about the concentration of immobile anions (A) make this impossible with the present data. The extrapolate of the experimental curve (Fig. 5) to less than zero concentration difference at zero oxygen uptake is to be expected from the Donnan equilibrium.

Two reasons can be suggested for the substrate experiments tending to show lower concentration differences for a given oxygen uptake: (i) the substrate supplied externally may introduce a number of single-step oxidations which are not as effective as the endogenous hydrogen transport in the accumulatory mechanism, and (ii) with substrate, the accumulation judged by chloride concentration may be less than the total accumulation because substrate anions may be competing with the chloride. Cleland's quoted results show that there is little difference between the rates of equilibration of chlorides and organic acid anions in sarcosomes, and Bartley and Davies (1954) have shown that the organic acid anions are accumulated by kidney mitochondria.

Thus mitochondria can be interpreted as having a high concentration of immobile anions responsible for the higher concentrations of mobile cations in the particle than in the external solution. Simultaneously an anion accumulation mechanism may operate across a membrane of high resistance. The concentration of anions thus maintained is consistent with that expected if the leakage through the membrane were balanced by an uptake of anions equivalent to the electron transport. The transport of anions would depend upon the cytochrome system, which, according to the arguments of Palade (1952), is probably located in the membrane. In such a position the cytochrome system may operate to transfer the anions across the membrane provided that the combination was so complete that an un-ionized complex was formed during the period of transport. This work indicates not only that the quantitative relations would be consistent with this hypothesis but that the anion accumulated is chloride, which is not recognized as entering directly into any of the known biochemical reactions of the mitochondria. The accumulation of the organic acid anions demonstrated by Bartley and Davies (1954) might be interpreted as being due to these anions being worked into the metabolic pool in which they participate, though accumulation may take place by the same mechanism as suggested here for chloride.

(d) Mitochondria and Salt Accumulation in Plants

The probable role of mitochondria in salt accumulation was discussed by Robertson (1951), who pointed out that the mitochondrion, if capable of accumulating ions, would be a suitable body to transfer ions across the cytoplasm and into lipid regions because not only do mitochondria have a lipoprotein structure but they are also moved effectively in the protoplasmic streaming. The results reported in this paper support this hypothesis. The mitochondrion, actively oxidizing substrate in one part of the cell, may increase its concentration of mobile ions and then, in cytoplasmic streaming, be moved to another part of the cell, where if its oxidative activity decreases, the ion concentration in the particle will also decrease. If the movement of the particle has been through a region of low permeability, the ions liberated from the particle will diffuse to the cell surface again only slowly. Such a low permeability could be due either to a high concentration of immobile ions in the cytoplasm, which would lower the diffusivity for Donnan reasons, or to a region of high lipid content. Microscopic examination of the cytoplasmic streaming shows that the mitochondria are in frequent contact with the inner layers of the cytoplasm.

Some recent work (Epstein and Hagen 1952; Epstein 1953; Scott Russell, Martin, and Bishop 1953) has concentrated on the hypothesis that carriers with some degree of specificity are concerned in combinations with the ions in the cytoplasm and their transport into the cell. If such carriers exist, their location in specific places in the cell is important and in this connection the present work on sodium and potassium distribution in mitochondria is interesting.

(e) Mitochondria and Electrolytes in Other Cells

The accumulatory capacity of mitochondria is probably important to tissues other than plant cells. The suggestions for the secretory activity of mitochondria in animal cells, discussed by Zollinger (1950), have been summarized recently by Lindberg and Ernster (1954). The present paper emphasizes the possibility that a definite ion transport mechanism exists in the mitochondria, related to the respiration and made possible by the membrane by which most mitochondria seem to be surrounded. Recent discussions of the gastric secretion of hydrochloric acid (Conway 1952; Davenport 1952) show that the quantitative relations are consistent with the hydrogen ions being derived from the hydrogens of respiration separated from the electrons which pass over the oxidase system. If the mitochondria of the secreting oxyntic cells have a membrane which is predominantly impermeable to cations and allows the electrons to pass in exchange for anions (e.g. Cl^-), the same secretory principle, dependent on mitochondrial structure and function, underlies both hydrochloric acid secretion and salt accumulation in plants.

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ENZYMIC ACTIVITY OF PARTICLES ISOLATED FROM VARIOUS TISSUES OF THE PEA PLANT

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Summary

A method is described for the preparation from green and etiolated tissues of the pea plant, of cytoplasmic particles with high oxidative activities in the presence of intermediates of the tricarboxylic acid cycle. The cofactor requirements have been investigated, and for preparations from etiolated pea stems, a phosphorylative ability comparable with animal mitochondria has been obtained. With certain of these preparations an oxidation of butyrate has been demonstrated.

I. INTRODUCTION

It has been shown that particulate preparations obtained from certain plant tissues contain an integrated system of enzymes, which can bring about the oxidation of intermediates of the tricarboxylic acid cycle and the subsequent synthesis of organic phosphate, the latter made possible by part of the energy released by these oxidations (Millerd *et al.* 1951). These particles were identified with the mitochondria present in the cytoplasm of intact plant cells. While cytological evidence has demonstrated the wide distribution of mitochondria in plant cells (see Newcomer 1951), the biochemical evidence of their ability to oxidize respiratory intermediates via the tricarboxylic acid cycle has been confined to relatively few tissues. Particulate preparations with this ability have been isolated from etiolated seedlings of mung bean (Millerd *et al.* 1951), pea (Davies 1953; Price and Thimann 1954), and lupin (Brummond 1952), the *Arum* spadix (Hackett and Simon 1954), potato tubers (Sharpensteen and Conn 1954), cauliflower buds (Laties 1953a), and the fruit of the avocado (Millerd, Bonner, and Biale 1953), and apple (Pearson and Robertson 1954). Little is known of the biochemical properties of such particles occurring in roots, while Brummond (1953) has cast doubt on the localization of the tricarboxylic acid cycle, as an integrated system, in cytoplasmic particles in the green leaves of lupins.

The purpose of this investigation was to demonstrate that biochemically active mitochondria could be isolated from various tissues of the one plant.

The results show that active mitochondria can be isolated from all major organs of both green and etiolated pea plants.

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II. METHODS

(a) *Plant Material*

(i) *Germinated Pea Seeds*.—Pea seeds *Pisum sativum* (Yates Greenfeast) were soaked in water for 4 hr, then in mercuric chloride (0.001M) for 2 min. The seeds were well rinsed with water, placed on moist cotton wool and kept for 2 days in the dark at 20-25°C. For each preparation 60 g of material was used.

(ii) *Pea Seedlings*.—Pea seeds were pretreated with water and mercuric chloride as described above, then planted in moist vermiculite. Etiolated seedlings were used after 1-2 weeks growth at 20-25°C, while green seedlings were grown at the same temperature in sunlight and used after 2-3 weeks growth. For each preparation 80 g of etiolated stems, 60 g of roots or green stems, 10 g of green leaves, or 5 g of etiolated leaves were used.

(iii) *Peas*.—These were obtained from local markets, 50-60 g being employed for each preparation.

(b) *Chemicals and Enzymes*

Coenzyme concentrate (Coe) was made from dried Brewer's yeast by adsorption of the coenzymes from an aqueous extract onto charcoal, followed by elution with 10 per cent. pyridine and precipitation with acetone, according to the method of Le Page and Mueller (1949). The Coe contained 15.5 per cent. diphosphopyridine nucleotide (DPN) and 0.8 per cent. triphosphopyridine nucleotide (TPN), these substances being assayed by the methods of Racker (1950) and Le Page and Mueller (1949) respectively. DPN (50 per cent.), TPN (30 per cent.), and glucose-6-phosphate dehydrogenase were prepared as described by Le Page and Mueller (1949), alcohol dehydrogenase according to Racker (1950),* and hexokinase according to Berger *et al.* (1946). Isocitrate was prepared by hydrolysis of the lactone (Krebs and Eggleston 1944). Ethylenediaminetetraacetate, adenylic acid (AA), and other substances employed, were obtained from commercial sources.

(c) *Preparation of Isolated Particles*

With the exception of pea shells, in which a Waring Blendor was employed, the plant tissue was ground in a mortar with 1/5 vol. of sand and 1/3 vol. of sucrose (0.5M) containing ethylenediaminetetraacetate (5×10^{-3} M), pH 7.3. The brei was strained through muslin and centrifuged at 1200 g for 3 min. The supernatant was further centrifuged at 15,000 g for 10 min. After decantation the residue was resuspended in 80 ml of the extraction medium and recentrifuged at 15,000 g for 10 min. The washing procedure was repeated using sucrose (0.5M) containing 10^{-3} M ethylenediaminetetraacetate. Finally the residue was resuspended in a minimum volume of sucrose (0.5M). 0.6 ml of this preparation contained from 0.4-0.8 mg total nitrogen. All steps of the preparation were carried out at 0-5°C.

* The TPN and alcohol and glucose-6-phosphate dehydrogenases were kindly supplied by Dr. G. Kellerman.

(d) Measurement of Activity

The oxygen consumption was measured using Warburg manometers, all experiments, unless otherwise stated, being conducted at 30°C. Except where phosphate uptake was also estimated, the substrate was placed in the main compartment and readings commenced after an initial equilibration period of 5 min. For the measurement of phosphate uptake the substrate was tipped after 7 min and oxygen uptake readings taken between 7 and 37 min. The reaction was stopped by tipping 0.2 ml of 40 per cent. HClO_4 from a second side arm. The reaction mixtures, including controls which contained no substrate, were quickly chilled, centrifuged, and the inorganic phosphate present in the supernatant estimated by the method of Allen (1940).

TABLE 1

THE EFFECT OF ETHYLENEDIAMINETETRAACETATE IN THE EXTRACTION MEDIUM ON THE OXIDATIVE ABILITY OF PARTICLES FROM VARIOUS TISSUES OF THE PEA PLANT

Reaction mixture contains enzyme (0.6 ml), sucrose (0.4M), MgSO_4 (0.001M), AA (0.0005M), Coe (1 mg), phosphate (0.01M), and α -ketoglutarate (0.02M). Final volume 2 ml

Material	Oxygen Uptake* ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)	
	Extraction Medium	
	Sucrose (0.5M)	Sucrose (0.5M) containing Ethylenediaminetetraacetate ($5 \times 10^{-3} \text{ M}$)
Green leaf	98	117
Green stem	117	230
Root	18	60
Etiolated leaf	128	262
Etiolated stem	36	295
Germinated seed	72	146
Fresh pea seed	180	211
Hull	82	180

* Corrected for endogenous oxygen uptake.

III. RESULTS

(a) The Oxidation of α -Ketoglutarate

The oxidative abilities of particulate preparations obtained from the various parts of the pea plant tested are shown in Table 1. Since it was thought that substances contained in the vacuole might have some injurious effect on the enzymic capacity of the particles, ethylenediaminetetraacetate was added in the hope that it might protect the particles from some of these substances (cf. Slater and Cleland 1953). Results showed that this procedure increased the activity in every case and ethylenediaminetetraacetate was hence included in the extraction medium for all subsequent experiments.

(b) *Cofactors*

The cofactor requirements for the various preparations are shown in Table 2. Magnesium ions and AA are essential if good activities are to be obtained. In general, cytochrome *c* and DPN have little, if any, effect on the activity, but the coenzyme concentrate from yeast stimulates the rate of oxidation. In the case of etiolated stems, this stimulation is not due to DPN or TPN (Table 3).

(c) *Oxidative Phosphorylation*

The requirement for AA suggests that phosphorylation concomitant with the oxidation is taking place and this was verified directly for etiolated pea stems and green leaves by measuring the uptake of inorganic phosphate during active oxidation (Table 4). Hexokinase and glucose were used to 'trap' the

TABLE 2

COFACTOR REQUIREMENTS FOR THE OXIDATION OF α -KETOGlutARATE BY PARTICLES FROM VARIOUS TISSUES OF THE PEA PLANT

The complete system contains enzyme (0.6 ml), sucrose (0.4M), phosphate (0.01M), MgSO_4 (0.001M), AA (0.0005M), cytochrome *c* (0.000005M), and α -ketoglutarate (0.02M). Final volume 2 ml

Material	Oxygen Uptake* ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)					
	-AA	-Mg	-Cytochrome <i>c</i>	Complete	Complete + DPN (0.001M)	Complete + Coe (1 mg)
Green leaf	22	19	140	139	137	221
Green stem	21	75	171	177	171	235
Root	28	14	49	47	40	244
Etiolated leaf	33	33	162	167	233	262
Etiolated stem	266	193	304	304	309	354
Germinated seed	27	31	59	81	79	111
Fresh pea seed	47	28	106	107	108	112

* Corrected for endogenous oxygen uptake.

esterified phosphate. Since the points of localization of phosphate esterification during the electron transport have not been determined, it is interesting to note that a phosphorylation also occurs when reduced DPN or TPN is used as substrate (Table 5). This was demonstrated by the addition of excess amounts of enzyme systems that reduce DPN and TPN respectively, namely the alcohol dehydrogenase and glucose-6-phosphate dehydrogenase systems.

(d) *The Oxidation of Other Substrates of the Tricarboxylic Acid Cycle*

With all the tissues tested, particulate preparations can be prepared, which will bring about the oxidation of pyruvate, isocitrate, glutamate, α -ketoglutarate, succinate, and malate (Table 6).

(e) The Oxidation of Butyrate

The oxidation of a fatty acid has not previously been demonstrated in plant mitochondria and the apparent lack of a system oxidizing fatty acids, ultimately via the tricarboxylic acid cycle and the cytochrome system, is the only major difference between mitochondria from animal and plant sources. Table 7 shows that butyrate can be oxidized by certain parts of the pea plant (green seedlings were not tested), but the oxidation is not easy to demonstrate. This is because of the apparent instability of the system. While the initial rate of

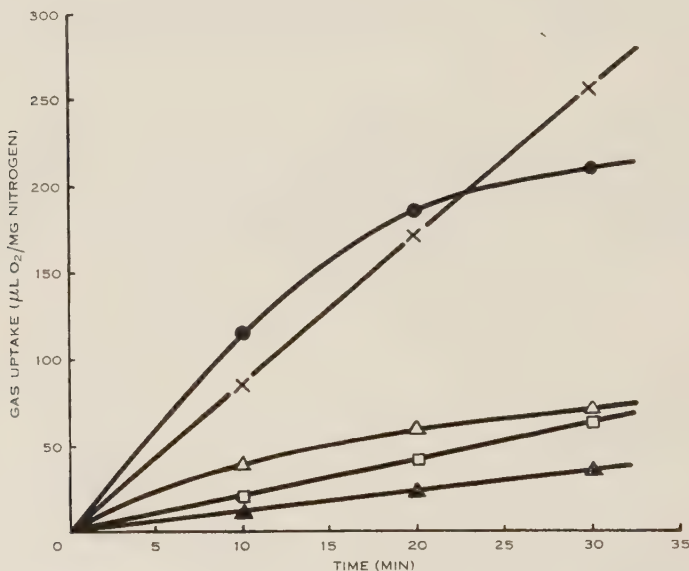


Fig. 1.—Rate of butyrate oxidation by particles from etiolated pea seedlings. Curves show the rate of oxygen uptake in the absence of added substrate (\blacktriangle), with DL-malate (0.003M) (\square), butyrate (0.05M) (\triangle), DL-malate (0.003M) + butyrate (0.05M) (\bullet), and α -ketoglutarate (0.02M) (\times). Reaction mixture also contains enzyme (0.6 ml), sucrose (0.5M), phosphate (0.01M), MgSO_4 (0.001M), and AA (0.0005M).

oxidation is high (comparable to that of α -ketoglutarate), the rate soon falls to the level of the sparker oxidation, usually within 15 min and at the most 25 min. A typical rate curve for butyrate oxidation with particles isolated from the aerial portion of etiolated pea seedlings is shown in Figure 1. It can be seen that the presence of a small amount of tricarboxylic acid cycle substrate (in this case malate), greatly enhances butyrate oxidation.

(f) The Duration of the Oxidative Ability of the Particles

The oxidation of succinate, α -ketoglutarate, isocitrate, and glutamate was usually linear for at least one hour and in some cases for 2-3 hr. Notable

TABLE 3

THE EFFECT OF DPN, TPN, AND COE ON THE OXIDATION OF α -KETOGLUTARATE: ETIOLATED PEA STEMS

The reaction mixture also contains enzyme (0.6 ml), sucrose (0.4M), phosphate (0.01M), MgSO_4 (0.001M), AA (0.0005M). Final volume 2 ml

Reaction Mixture	Oxygen Uptake ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)
Enzyme	2
Enzyme + α -ketoglutarate	282
Enzyme + α -ketoglutarate + DPN (0.001M)	301
Enzyme + α -ketoglutarate + TPN (0.001M)	282
Enzyme + α -ketoglutarate + DPN + TPN	305
Enzyme + α -ketoglutarate + Coe (1 mg)	384

exceptions were the preparations obtained from root material, where the oxidation of all substrates was not linear and had generally ceased in 20 to 40 min. Further, the oxidation of pyruvate was not always linear and in most prepara-

TABLE 4

THE P : O RATIO FOR α -KETOGLUTARATE AND SUCCINATE

The reaction mixture contains enzyme (0.6 ml), sucrose (0.4M), glucose (0.02M), phosphate (0.04M), MgSO_4 (0.001M), AA (0.0005M), Coe (1 mg), and substrate (0.02M). Final volume 2 ml

Preparation	Number	Light or Dark	Substrate	Additions	Phosphorus Uptake (atoms $\times 10^{-6}/30 \text{ min}$)	Oxygen Uptake (atoms $\times 10^{-6}/30 \text{ min}$)	P : O
Etiolated pea stems	1		α -Ketoglutarate	—	0.0	12.3	—
	1		α -Ketoglutarate	Fluoride (0.1M)	1.9	4.0	0.47
	2		α -Ketoglutarate	Hexokinase (0.2mg)	36.1	12.6	2.86
	3		α -Ketoglutarate	Hexokinase (0.2 mg)	50.1	16.2	3.10
	4		Succinate	Hexokinase (0.2 mg)	35.5	24.5	1.45
	5		Succinate	Hexokinase (0.2 mg)	30.2	24.8	1.22
Green leaves (temp. 15°C)	6	Light *	Succinate	Hexokinase (0.2 mg)	11.9	8.4	1.42
	6	Dark	Succinate	Hexokinase (0.2 mg)	11.0	7.7	1.48

* Under the conditions of light and temperature used in this experiment, similar preparations from green pea leaves gave a photolytic activity of 600-800 $\mu\text{l O}_2/\text{mg chlorophyll/hr}$, using the ferricyanide assay method of Arnon and Whatley (1949).

tions fell off with time (cf. Laties 1953*b*). Where tried, the oxidation of citrate and fumarate proceeded at a rate comparable with that of glutamate.

IV. DISCUSSION

Studies on the oxidative ability of isolated cytoplasmic particles from pea plants have been confined to the stems of etiolated plants (Davies 1953; Price and Thimann 1954). The activities reported here compare more than favourably with those previously obtained, but under the conditions used here, high activities could be obtained only if ethylenediaminetetraacetate was included in the extraction medium. A similar, though less striking effect, was obtained in all other tissues tested, and it would appear likely that the ethylenediamine-tetraacetate protects the particles from some injurious substances, released from the cell vacuole.

TABLE 5
OXIDATIVE PHOSPHORYLATION WITH DPN AND TPN: ETIOLATED PEA STEMS

Substrate	Phosphorus Uptake (atoms $\times 10^{-6}$ /30 min)	Oxygen Uptake (atoms $\times 10^{-6}$ /30 min)	P : O
DPN*	5.2	5.3	0.98
TPN†	7.2	13.8	0.57

* Complete reaction mixture contains enzyme (0.6 ml), sucrose (0.4M), phosphate (0.02M), glucose (0.02M), hexokinase (0.2 mg), alcohol (0.2M), alcohol dehydrogenase (1 mg), DPN (0.001M), MgSO_4 (0.001M), and AA (0.0005M). Final volume 2 ml.

† Complete reaction mixture contains enzyme (0.6 ml), sucrose (0.4M), phosphate (0.02M), glucose (0.02M), hexokinase (0.2 mg), glucose-6-phosphate dehydrogenase (1 mg), TPN (0.001M), MgSO_4 (0.001M), and adenosine triphosphate (0.001M). Final volume 2 ml.

The cofactor requirements are similar to those needed for comparable preparations from both animal and plant tissues, although the reason for the additional stimulation by the coenzyme extract is obscure. It is not due to any lack of DPN (Table 2), nor in etiolated pea stems can it be attributed to the lack of TPN (Table 4). Further, the stimulation is probably not due to coenzyme A present in the Coe, since a stimulation of approximately the same magnitude occurs when succinate is the substrate. These considerations, together with the fact that the AA requirement shows that the oxidative rate is limited by the rate of phosphorylation (cf. Laties 1953*b*) suggest that the site of action of the Coe effect is somewhere along the pathway of phosphorylation.

Hitherto, it has not been possible to demonstrate phosphorus : oxygen (P : O) ratios in particulate preparations from plants comparable with those observed in preparations from animal tissues (Biale 1953; Bonner and Miller 1953). Although a P : O ratio greater than unity was not obtained for the oxidation of reduced DPN and TPN, the P : O ratios obtained with succinate and α -ketoglutarate are comparable with those obtained with isolated animal mitochondria, and it would appear justified to assume a phosphorylation quotient of

TABLE 6

OXIDATION OF SUBSTRATES OF THE TRICARBOXYLIC ACID CYCLE BY PARTICLES FROM VARIOUS TISSUES OF THE PEA PLANT

Composition of the reaction mixture as in Table 1. Substrate concentration 0.02M except DL-malate (0.0015M)

Material	Oxygen Uptake ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)						
	No Substrate	Malate	Malate + Pyruvate	α -Keto-glutarate	isoCitrate	Succinate	Glutamate
Green leaf (i)	16	—	—	182	116	232	91
(ii)	—	25	256	—	—	—	—
Green stem	55	72	159	342	213	562	256
Root (i)	17	—	—	330	73	164	78
(ii)	—	45	193	—	—	—	—
Etiolated leaf	7	100	286	350	306	598	229
Etiolated stem	0	69	380	405	254	747	384
Germinated seed	50	59	404	277	172	372	188
Fresh pea seed	17	22	142	110	86	210	127
Hull	48	104	260	240	—	168	156

2 for the reaction succinate \longrightarrow fumarate, and of 4 for the reaction α -ketoglutarate \longrightarrow succinate (cf. Krebs 1954). It is interesting to note that light has no significant effect on the rate of oxidation or phosphorylation in preparations containing both mitochondria and whole chloroplasts (Table 3).

The oxidation of butyrate merits some consideration. Previously Newcomb and Stumpf (1952) demonstrated the oxidation of palmitate by cell free preparations of peanut cotyledons. In particular, palmitate, but not any of the

TABLE 7

THE OXIDATION OF BUTYRATE BY PARTICLES FROM VARIOUS TISSUES OF THE PEA PLANT

The reaction mixture except for substrates is as in Table 1

Material	Oxygen Uptake ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)	
	Malate	Malate + Butyrate
Etiolated stem*	37	90
Etiolated first leaf*	57	162
Root*	78	129
Germinated seed†	59	180
Fresh pea seed†	28	97

* DL-Malate (0.003M), butyrate (0.02M).

† DL-Malate (0.0015M) and butyrate (0.05M).

lower fatty acids, was oxidized by a cell fraction containing particles of microsomal size, but no oxidation of fatty acids occurred with the mitochondrial fraction. While it is true that the particulate fraction, prepared as already described, would probably contain the larger microsomes, as well as the still larger mitochondria, the initial high rate of butyrate oxidation indicates that this oxidation is taking place on the same particles which bring about the oxidation of the tricarboxylic acid cycle substrates. Both processes are in some way related since small amounts of malate appreciably increase the rate of butyrate oxidation, and it hence appears fairly certain that enzymes contained in the mitochondria are essential for this oxidation, although the possible participation of microsomal particles cannot be discounted. Further work is proceeding towards a more positive identification of the site of butyrate oxidation.

V. CONCLUSION

The above results indicate that the enzymes of the tricarboxylic acid cycle are universally distributed in the cells of the pea plant and that these enzymes are located as a complete integrated system on sub-cellular particles known as mitochondria. The high oxidative activities obtained, together with the demonstration of P:O ratios comparable with those obtained with animal mitochondria during the oxidation of α -ketoglutarate and succinate, lead to the conclusion that the tricarboxylic acid cycle-cytochrome system is the major respiratory mechanism operating in the cells of either etiolated or actively photosynthesizing pea plants.

VI. ACKNOWLEDGMENTS

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THE RESPONSE OF THE YOUNG TOMATO PLANT TO A BRIEF PERIOD OF WATER SHORTAGE

I. THE WHOLE PLANT AND ITS PRINCIPAL PARTS

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Summary

The growth response was determined of young tomato plants subjected to wilting treatments of short duration. The experiments were conducted in pots in the glasshouse using Jondaryan loam, and the wilting treatments were at a "moderate" and a "severe" level. Even with the severe treatment, soil water did not fall below the permanent wilting percentage.

Both wilting treatments reduced growth during the period of wilting, but growth rates upon re-watering were greater than for the control plants. There was no indication that this recovery effect was complete at the final harvest.

During wilting, a higher stem weight ratio and a lower lamina weight ratio were developed than in the control, and relative growth rates and net assimilation rates were depressed in both treatments. After wilting, lamina weight ratios became higher than control, stem weight ratios became lower, and net assimilation rates and relative growth rates rose above control values.

These treatment effects may be interpreted as a tendency towards senescence during wilting and a return to a more juvenile condition upon re-watering.

It was concluded that the changes in weight ratios were due to modifications of the normal pattern of translocation between plant parts. It was also concluded that the changes occurring in response to the water shortage were initiated relatively early in the drying cycle.

The effects of water shortage on the water economy of the plant were also considered. It was found that, whilst there was a high efficiency of transpiration during wilting, the drastic effects of the water shortage on growth made the greater efficiency of doubtful merit. After wilting, there was no economy in water usage by plants of either of the wilt treatments.

I. INTRODUCTION

The effects of water shortage on the growth and yield of plants have been the subject of much study, and the relevant literature has been reviewed by Veihmeyer and Hendrickson (1950) and by Richards and Wadleigh (1952). The last two authors have drawn attention to the difficulties of imposing low water treatments which are objective in character. The best that can be achieved is to set lower and upper limits to the levels of soil water within any specified treatment. Most workers have imposed low water treatments that continued for a considerable part of the growing period, but in the data presented here the treatment consists of a relatively short period of wilting imposed between periods of normal moisture relationships. This was done in order to

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obtain information that was free from the complicating effects of repeated water shortages and could thus be more readily interpreted. Petrie and Arthur (1943) included temporary low water supply in their treatments when considering the effects of varying water supply on the growth of tobacco. They found this treatment gave a significant increase in the final dry weights of their plants.

Goodall (1945, 1946) has considered the distribution of weight change in the young tomato plant when at the eight-leaf stage, that is, with the eighth leaf about 1 cm long. A similar stage of growth was chosen on this occasion for the beginning of the wilting treatments so that comparisons with Goodall's work could be made where relevant. At this stage the eighth leaf had a mean length of 5 cm and a mean area of 10.2 sq. cm. and was sufficiently large to be separated into lamina and petiole.

II. EXPERIMENTAL

(a) General

The experiments were conducted in the glasshouse. Seeds of the tomato (*Lycopersicon esculentum* Mill. var. Pearson) were sown in containers holding 3.2 kg oven-dry soil. Fruit cans without drainage were used as pots and were surrounded by paper jackets to protect the sides from direct insolation. Jon-daryan loam (Taylor and Hooper 1938), representative of the cultivated layer, was used and was crushed to pass a $\frac{1}{8}$ in. mesh. It contained 180 p.p.m. phosphorus as dilute acid soluble P_2O_5 by the method of Truog (1930), 12 p.p.m. ammonia nitrogen, and 52 p.p.m. nitrate nitrogen. The moisture content at field capacity (F.C.) was 22 per cent. as determined by sticky points, and at the permanent wilting percentage (P.W.P.) it was 11.1 per cent. as determined by the sunflower test of Veihmeyer and Hendrickson (1949, pp. 79-81). A layer of quartz gravel 0.5 in. deep was spread on the surface of the soil to reduce evaporation. Until treatments were begun, all pots were maintained at field capacity by frequent waterings to the required weight.

The water added to each pot was recorded from the time of commencing treatment until the final harvest. Temperature and humidity records were kept in the glasshouse, and records of hours of bright sunshine were taken from the meteorological data recorded nearby. The plants were thinned to one per pot and axillary shoots were removed as they appeared. Total leaf area was determined at various stages of growth, using the method of Williams (1954). Treatments were allotted at random within size groups determined on the basis of leaf area estimations.

Two experiments were conducted, in different years, but at the same time of the year and in the same glasshouse. The second experiment was designed to amplify the findings of the first on a more rigidly controlled basis and to provide more material for chemical analyses. Replication was therefore greater and great care was taken to obtain maximum uniformity because of the effect leaf area has on water loss. A pure line seed was developed for this purpose from discarded plants of experiment 1. The plants were grown in separate

portions of the same soil sample in each experiment, but nutrient additions differed in the two experiments, as shown below.

Treatments are represented diagrammatically in Figure 1, where the changes in soil water for the two treatments are plotted against time for the two experiments. It was necessary to devise these two treatments in such a way that they began together and ended together, otherwise comparisons during the recovery period would be meaningless. The more moderately treated plants

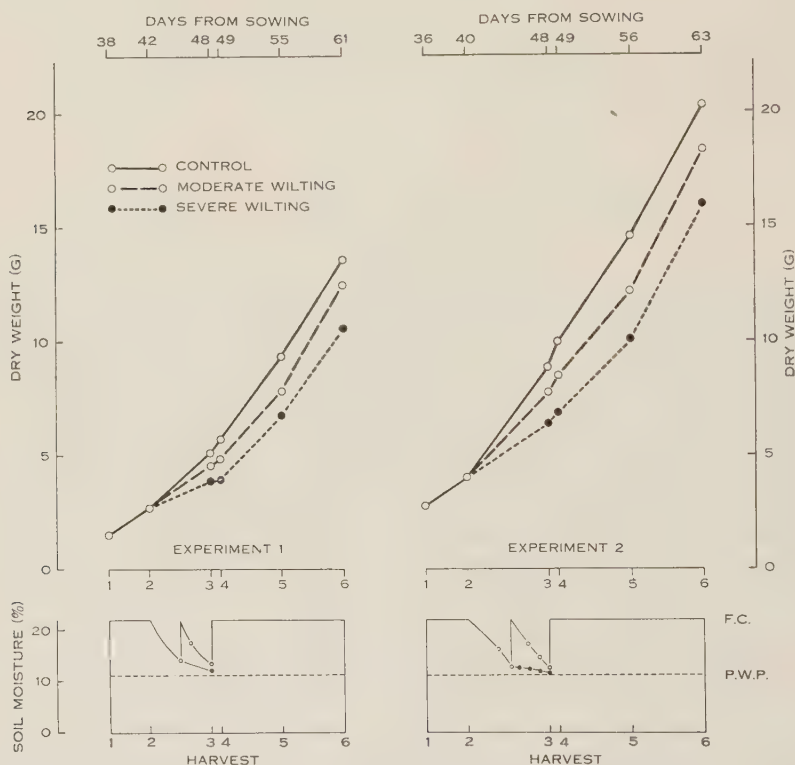


Fig. 1.—Dry weight of whole plant and diagram of soil moisture regime. F.C., field capacity; P.W.P., permanent wilting percentage, as determined by standard sunflower test.

were re-watered on two occasions, on each of which the plants lost turgor in bright sunshine but readily revived in the shade. Plants receiving the severe treatment were re-watered only once, and at this time the average soil water had been reduced almost to the P.W.P. and the leaves were wilted all day (see Plate 1). The timing for this sequence was worked out in advance with a small pilot experiment. It is important to note that soil water was at no time below the P.W.P. Soil water levels at the end of the wilting period were determined by pot weighings, allowing for the fresh weights of the plants. The results were checked by direct determinations of soil water in pots whose plants were harvested at that time. In the second experiment, the plants were observed each morning at dawn. At this time plants of the severe treatment had

regained turgor, except on the morning of the day when all plants were watered to F.C. The glasshouse was maintained at higher humidity throughout experiment 2 than in experiment 1 and the temperature was lower, so that loss of moisture was more gradual. The wilting period was therefore 2 days longer in experiment 2 than in experiment 1.

Early on the morning of harvest, the soil was brought to field capacity, except when the plants were harvested in the wilted condition. The plants were taken to the laboratory before 9 a.m. The tops were removed at ground level and separated into the fractions enumerated below, the operations being performed on a block basis and extending throughout the day. Fresh weights were determined with negligible loss of moisture and the fractions were dried at 80°C. The roots were then washed free of soil by the method of Gates (1951).

(b) Details of Experiments

Experiment 1.—Day 0: March 21, 1947; harvests: days 38, 42, 48, 49, 55, 61; fractions separated: cotyledons; lamina and petiole, separately, of each of the first eight leaves; laminae and petioles, as separate groups, of any remaining leaves; stem; roots. The inflorescences and any leaves too small to separate were placed in the stem fraction. The laminar fractions comprised the blades of the leaflets separated at their junction with the petiolules. The petiolar fractions thus include both the main axis and the petiolules. Total leaf area determinations: days 35, 38, 42, 49, 54, 61. Nutrient applications: day 31, 0.5 g KH_2PO_4 and 1.0 g $(\text{NH}_4)_2\text{SO}_4$ per pot. Replication: 8.

Experiment 2.—Day 0: March 25, 1949; harvests: days 36, 40, 48, 49, 56, 63. Removal to the laboratory and beginning of harvesting was delayed for 4 hr on day 49. Fractions separated: lamina and petiole, separately, of the second and of the sixth leaf; laminae and petioles, as separate groups, of remaining leaves below the fifth; laminae and petioles, as separate groups of remaining leaves above and including the fifth; stem; roots; and, on day 63, the inflorescences. The fractions were constituted in the same manner as the corresponding parts in experiment 1, except that the stem does not include the inflorescences at day 63. Total leaf area determinations: days 26, 32. Nutrient applications: days 30, 51, 58. Rates as for experiment 1. Replication: 14.

III. RESULTS

Treatments will be referred to in the following manner: Control (C): frequent watering to field capacity; WM: brief period of moderate wilting; WS: brief period of severe wilting. Treatment effects of wilting will be seen to have occurred both during and following the actual water shortage, and will therefore be referred to as effects during and after wilting.

(a) Primary Data

(i) Dry Weight of Whole Plant

The dry weight curves for the whole plant are shown in Figure 1 and the numerical values are given in Table 1. The soil moisture trends are shown below the respective curves.

TABLE 1
 DRY WEIGHTS (G) OF WHOLE PLANT AND PRINCIPAL PARTS*

Plant Part	Harvest	Experiment 1 (improved estimates)				Experiment 2 (experimental values)			
		Day	Control (C)	Moderate Wilting (WM)	Severe Wilting (WS)	Day	Control (C)	Moderate Wilting (WM)	Severe Wilting (WS)
Whole plant	1	38	1.49			36	2.76		
	2	42	2.69			40	3.99		
	3	48	5.12	4.55	3.86	48	8.83	7.71	6.36
	4	49	5.71	4.82	3.95	49	9.94	8.43	6.82
	5	55	9.30	7.80	6.74	56	14.55	12.16	10.08
	6	61	13.57	12.43	10.55	63	20.28	18.38	15.97
Total laminae	1	38	0.78			36	1.44		
	2	42	1.30			40	1.94		
	3	48	2.26	1.88	1.63	48	3.92	3.27	2.72
	4	49	2.50	2.00	1.67	49	4.39	3.68	3.04
	5	55	3.53	3.04	2.76	56	5.49	4.68	4.09
	6	61	4.50	4.18	3.84	63	7.17	6.49	6.07
C v. WM at day 61, $P < 0.05$									
Petioles	1	38	0.24			36	0.47		
	2	42	0.46			40	0.77		
	3	48	0.94	0.85	0.73	48	1.89	1.63	1.33
	4	49	1.05	0.89	0.74	49	2.09	1.78	1.42
	5	55	1.68	1.38	1.21	56	3.07	2.48	2.03
	6	61	2.38	2.08	1.76	63	3.78	3.34	2.96
Stem (including inflorescence when present)	1	38	0.24			36	0.44		
	2	42	0.54			40	0.71		
	3	48	1.22	1.23	0.97	48	1.95	1.83	1.47
	4	49	1.38	1.29	1.06	49	2.17	1.89	1.41
	5	55	2.83	2.23	1.77	56	4.03	3.40	2.56
	6	61	5.13	4.62	3.61	63	6.87	6.33	5.05
Comparisons at day 48, n.s.; C v. WM at day 49, n.s.									
C v. WM at day 48, n.s.; C v. WM at day 63, $P < 0.05$									
Inflorescence	6	61	—	—	—	63	2.42	2.50	1.86
							C v. WM, n.s.; C v. WS, $P < 0.05$		
Roots	1	38	0.22			36	0.41		
	2	42	0.38			40	0.58		
	3	48	0.71	0.59	0.52	48	1.06	0.98	0.85
	4	49	0.77	0.64	0.48	49	1.29	1.08	0.95
	5	55	1.25	1.13	1.00	56	1.96	1.61	1.40
	6	61	1.56	1.54	1.30	63	2.45	2.22	1.89
WM v. WS at day 48, $P < 0.05$; C v. WM at day 55, $P < 0.05$; WM v. WS at day 55, n.s.; C v. WM at day 61, n.s.									
C v. WM at day 48, n.s.; WM v. WS at day 49, $P < 0.05$; C v. WM at day 63, $P < 0.05$									

* Comparisons at time of harvest are significant at the level, $P < 0.01$ or lower unless otherwise stated. n.s.: not significant.

The data for experiment 1 are improved estimates of experimentally determined values, but the data for experiment 2 are experimental means. This difference in procedure was necessitated by a difference in the range of plant size between the two experiments. The mean leaf area for both experiments just before harvest 1 was approximately 250 sq. cm., but the standard error for experiment 1 was 41.92 whereas it was only 14.38 for experiment 2. In view of this greater plant size variability in experiment 1, the procedure outlined by McIntyre and Williams (1949, p. 343) for gaining improved estimates of dry weight was adopted. In this case, the weights were referred back to the general mean of the pretreatment ratings, and the rating values were regarded as fixed for repeated sampling.

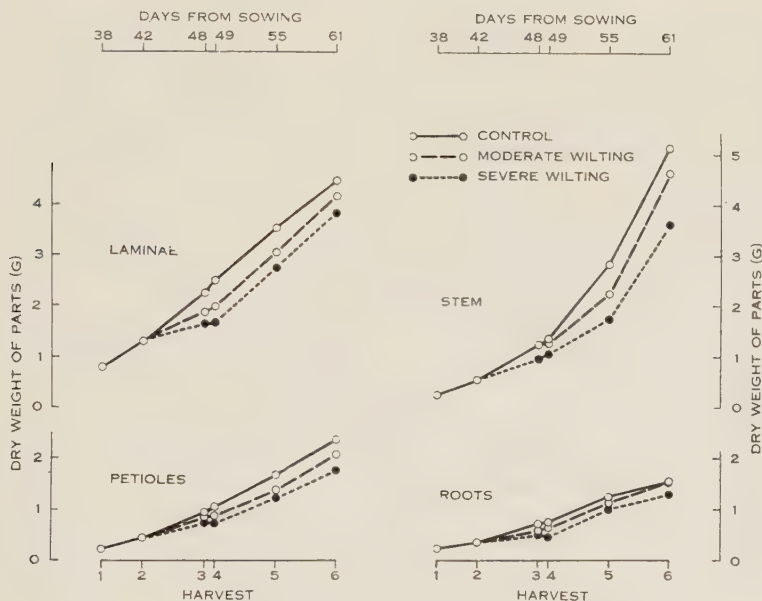


Fig. 2.—Dry weight of principal plant parts for experiment 1 only. The stem fraction includes inflorescences.

The curves for whole plant dry weight show a marked similarity in the two experiments, except that the plants of experiment 2 are bigger at comparable ages than are those of experiment 1. The periods of water deficiency between harvests 2 and 3 in both experiments, markedly retarded the growth of the plants, even though soil moisture did not drop below the permanent wilting percentage in WS, and, for most of the time, was well above this point in WM. Plate 1 shows the degree to which treatment affected the appearance of the plants at harvest 3, just before watering to field capacity. The dry weight curves show that the check to growth due to WM was about half that due to WS, although the nature of the treatments would not lead one to expect such a large effect with WM. In experiment 2, the dry weight curves for interval 3-4 are steeper than might have been expected, and this may have been contri-

buted to by the 4-hr delay in taking harvest 4. The general trends of the dry weight curves are very similar after harvest 4 and there is little evidence that the additional nutrients supplied in experiment 2 have influenced the treatment relations.

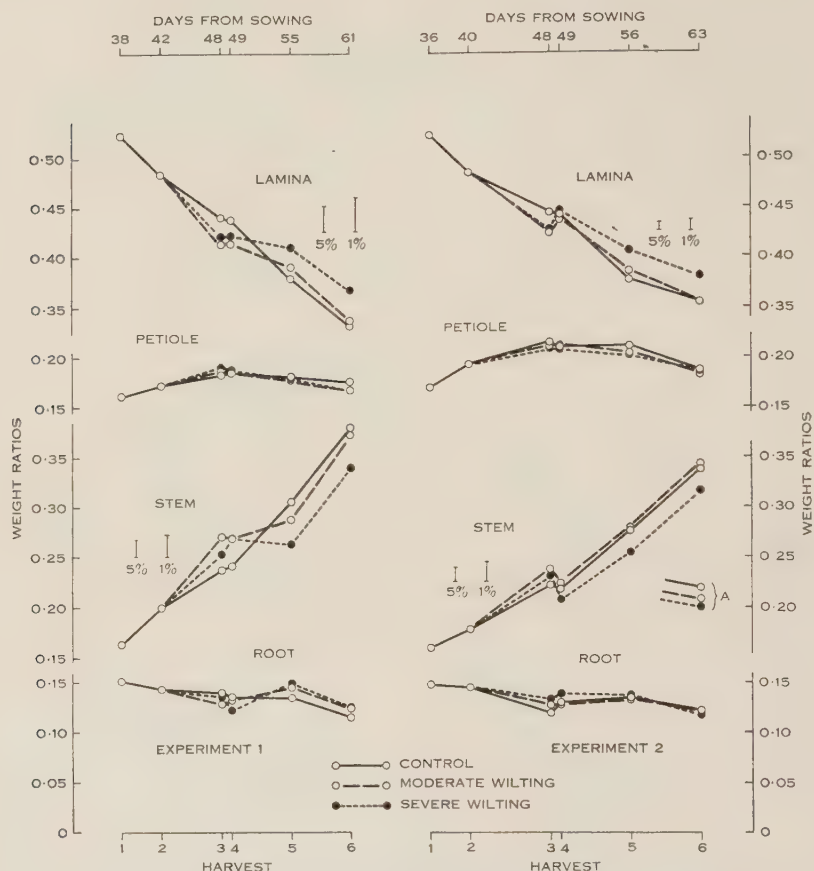


Fig. 3.—Weight ratios of principal plant parts. The stem fraction includes inflorescences. A, the ratios for stem minus inflorescences at harvest 6. Minimum significant differences for lamina and stem weight ratios are indicated in the usual way on the graph.

(ii) Dry Weights of Principal Parts

Dry weight data for laminae, petioles, stem, and roots are shown in Figure 2 for experiment 1 and in Table 1 for both experiments. That the rate of stem growth should appear to increase rapidly with time is due largely to the inclusion of the inflorescences with this fraction.

Wilting retarded development in each part of the plant, but the severity of the effect during wilting was greater in the laminae than in the stem. This differential effect of treatment was most pronounced in WM, in which the effect on stem weight was very small. Upon recovery from wilting the curves have different trends. The lamina weights tend to converge, but the curves for

the remaining parts, especially the stem, do not. The stem curves for all treatments diverge considerably during the interval 4-5, and continue to do so during the interval 5-6 for WS. The data for experiment 2 suggest that the effect of WS on stem development may be due in part to a delayed development of the inflorescence, for WS depressed inflorescence weight relatively more than that of stem minus inflorescence at the final harvest.

The parallel trends of the whole plant dry weight curves are seen to be a composite of changing relationships between the various principal fractions in response to treatment and, indeed, the weight ratios of plant parts show this to be so.

(b) *Weight Ratios of Plant Parts*

Figure 3 shows trends in the weight ratios of the plant parts for both experiments, and these provide indices of distribution of dry matter between these parts. At the beginning of the experiments more than half of the dry matter of the plant is found in the leaf laminae, but the lamina weight ratio falls steeply with time, and this is balanced by a rise in the stem weight ratio. This rise is accentuated by the inclusion of the inflorescence in that fraction, as can be seen from the graph for experiment 2 where A signifies stem minus inflorescence. The root weight ratio tends to fall slightly and the petiole weight ratio first increases and then decreases slightly during the experimental period.

Effects of treatment on the lamina and stem weight ratios are statistically significant. During wilting, the lamina weight ratio decreases and the stem weight ratio increases, but upon returning to field capacity again this trend is reversed, and the lamina weight ratio increases relative to the control, whilst that for the stem decreases. The tendency is apparent in both experiments, but significance is more easily established in experiment 2 by reason of its greater precision. WM weight ratios exhibit the same trend as those for WS, but they depart significantly from the control only at harvest 3. The inclusion of the inflorescences with the stem tends to modify the stem pattern, for, in experiment 2, stem minus inflorescence in WM is below and not above the control at harvest 6.

(c) *Growth Analysis*

Figure 4 shows the curves for the relative growth rate R , net assimilation rate on a lamina dry weight basis E_w , and net assimilation rate on a lamina protein nitrogen* basis E_p . In order to simplify comparisons of trends between the three sets of curves, the scales for E_w and E_p data have been adjusted so that the means of the control values for each, in the two experiments, are equal to the comparable mean scale for R . This does not do violence to the data, as the controls have a similar drift with time and the ratios between them are similar for the two experiments. Harvest 3 has been ignored for the purpose of the growth analysis. Net assimilation rates on an area basis are similar to E_w values, so are not presented.

* The data for lamina protein nitrogen will be presented in a later paper.

(i) *Relative Growth Rate, R*

During wilting the relative growth rates are considerably and significantly reduced, as is to be expected from the effect of wilting on dry weight. Even the moderately wilted plants are affected in this manner in both experiments. Soon after the wilting period, R is restored to a level not differing significantly from that of the control. However, during interval 5-6, the R values rise significantly above the control. The significance attained is $P < 0.001$ for WS *v.* control, and $P < 0.01$ for WM *v.* control in experiment 2, whilst for corresponding comparisons in experiment 1, $P = 0.06$ and $P < 0.05$. These treatment relations are essentially the same in the two experiments for interval 5-6, even though additional nutrient was given in experiment 2.

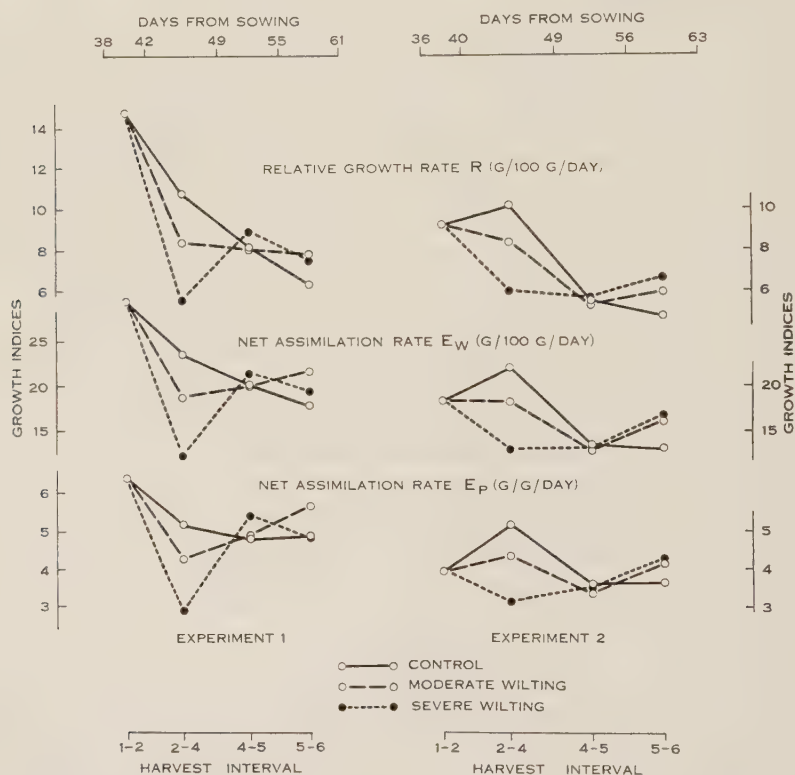


Fig. 4.—Growth indices for the whole plant. R , relative growth rate on a whole plant dry weight basis; E_w , net assimilation rate on a lamina dry weight basis; E_p , net assimilation rate on a lamina protein nitrogen basis.

(ii) *Net Assimilation Rate*

The curves for E_w are similar to those for R , because the leaf weight ratio, the other component of R , does not show marked departures due to treatment. The treatment depressions in E_w for interval 2-4 are again highly significant. None of the differences for interval 4-5 are significant, but at interval 5-6 the

departures from control are more significant for experiment 2: the level of significance for WM being $P < 0.01$ and for WS, $P < 0.001$. In experiment 1, the comparison WM *v.* control almost attains significance. The two experiments show essentially similar trends in response to wilting in that a marked fall in net assimilation rate during the water shortage is followed by a trend to higher values than those for control at a relatively short time after re-watering.

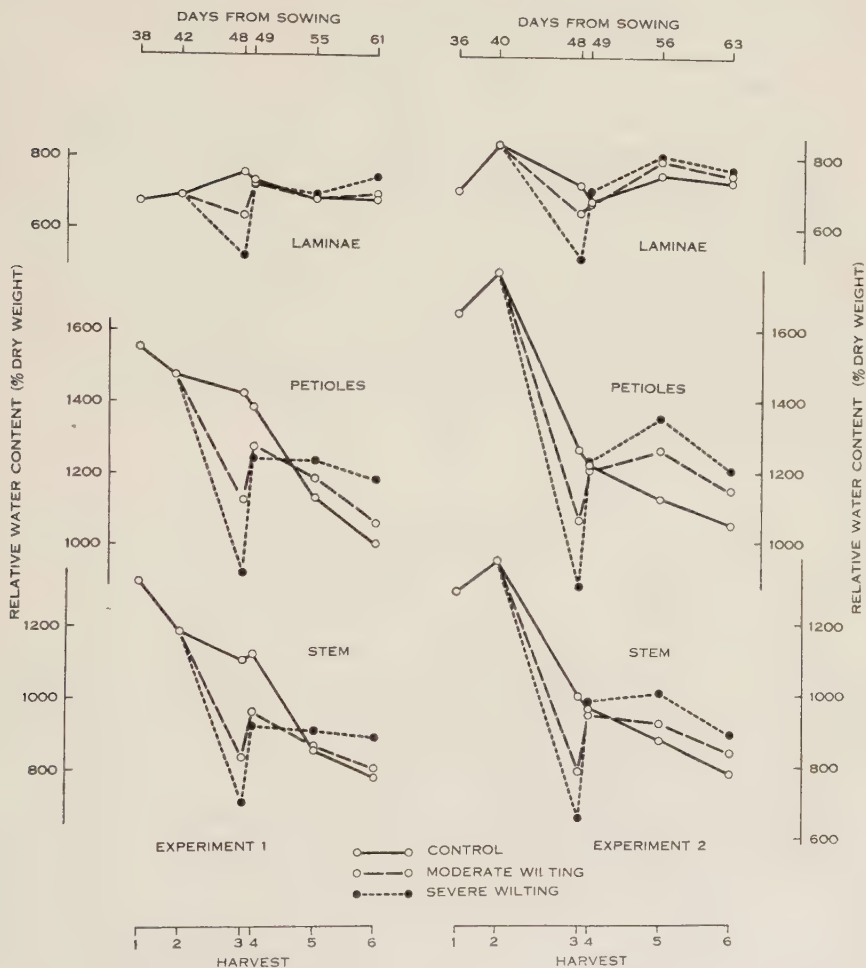


Fig. 5.—Relative water content of principal plant parts as per cent. dry weight.

The curves for E_p are very similar to those for E_w , though the general trends are slightly different. This difference is due to the normal tendency for lamina protein contents to fall with time. There are small but real differences in treatment effect on E_p after wilting compared with those on E_w . These are most marked in experiment 1, and are caused by effects of treatment on lamina protein contents.

The fact, already mentioned, that harvest 4 had to be delayed 4 hr, would have an appreciable effect on the time trends in R , E_w , and E_p for experiment 2, but would not affect the treatment comparisons.

(d) Plant Water

The absolute water contents of the plant tops and of the laminae, petioles, and stem are presented in Table 2. The water contents per cent. dry weight for these parts are shown graphically in Figure 5, for both experiments.

As is to be expected, the absolute and relative water contents of the parts of the wilted plants were markedly less than those of the control. In both experiments, and especially in the second, the tops of the WS plants had less water in them at the conclusion of the wilting (harvest 3) than at the beginning (harvest 2), even though they had increased in dry weight during this period. This tendency towards a net loss of water during the wilting period was greatest in the laminae. With WM there was a net gain in the absolute water content of the tops during wilting, but the relative water contents of the parts fell significantly below those of the control. Comparisons of treatment effect on the relative water contents of the plant parts when wilted are difficult because different parts of the plant have very different relative water contents. It is perhaps helpful to express the effects at harvest 3 as percentages of the control values in each plant part:

Experiment:		1	2	1	2	1	2
		Laminae		Petioles		Stem	
Treatment	WM:	83.3	89.0	79.0	84.1	75.1	78.8
	WS:	69.0	70.9	64.6	69.4	64.2	65.6

The drying of the tissues during wilting is to a similar extent in the two experiments, and the depression relative to control is least in the laminae and greatest in the stem. This implies that the laminae of the wilted plants were relatively more succulent than the stems, and this occurs in spite of the tendency noted above towards a greater net loss of water from the laminae.

As might be expected, upon returning to field capacity at the conclusion of wilting, there is a rapid uptake of water. Thereafter, the absolute water content of the wilted plants does not overtake that of the control plants, but the values converge for the laminae and the petioles in both experiments and diverge slightly in the stem. The response to WM is intermediate to that for WS and the control, and all comparisons are highly significant in both experiments.

After the wilting period, the curves for relative water content in the laminae, petioles, and stem cross over to positions which have an inverse relationship to their trend during wilting. Whereas WS causes the more severe depression during wilting, upon recovery it gives rise to plants with a higher relative water content than those of WM. Differences for all parts are significant at harvest 6, except the comparison WM *v.* control in experiment 1 for stem and for laminae. The levels of significance are high at harvest 5. Not all differences are signifi-

cant at harvest 4, but controls are above WM and WS in experiment 1 for petioles and stems. The change in relative water content upon re-watering appears to occur earlier in experiment 2 than in experiment 1. This may have

TABLE 2
ABSOLUTE WATER CONTENT (G) OF PLANT PARTS*

Plant Part	Harvest	Experiment 1				Experiment 2			
		Day	Control (C)	Moderate Wilting (WM)	Severe Wilting (WS)	Day	Control (C)	Moderate Wilting (WM)	Severe Wilting (WS)
Tops	1	38	12.6			36	23.6		
	2	42	22.5			40	39.5		
	3	48	44.0	31.7	22.0	48	72.2	53.0	35.2
	4	49	48.4	38.5	31.2	49	76.3	64.9	53.0
	5	55	67.5	56.6	51.4	56	110.9	99.8	86.4
	6	61	94.3	88.4	80.6	63	145.9	140.6	127.5
Laminae	1	38	5.4			36	10.2		
	2	42	9.1			40	16.2		
	3	48	17.2	11.9	8.6	48	28.5	21.1	14.0
	4	49	18.4	14.6	12.1	49	29.8	25.4	21.5
	5	55	24.1	20.8	19.2	56	41.1	37.1	32.9
	6	61	30.4	29.1	28.3	63	52.4	48.8	46.5
WM <i>v.</i> WS at day 61, n.s.									
Petioles	1	38	3.8			36	7.7		
	2	42	6.9			40	13.5		
	3	48	13.3	9.6	6.8	48	24.0	17.3	11.6
	4	49	14.6	11.4	9.2	49	25.5	21.6	17.5
	5	55	19.1	16.4	14.9	56	34.6	31.3	27.6
	6	61	23.8	22.1	20.6	63	39.6	38.5	35.8
WM <i>v.</i> WS at day 61, $P < 0.05$ C <i>v.</i> WM at day 63, n.s.									
Stem (including inflores- cence when present)	1	38	3.3			36	5.7		
	2	42	6.4			40	9.8		
	3	48	13.5	10.3	7.0	48	19.7	14.5	9.6
	4	49	15.4	12.5	9.8	49	20.9	17.9	13.9
	5	55	24.4	19.5	16.1	56	35.2	31.4	25.9
	6	61	40.0	37.2	31.6	63	53.9	53.3	45.2
C <i>v.</i> WM at day 61, n.s.; WM <i>v.</i> WS at day 61, $P < 0.05$ C <i>v.</i> WM at day 61, n.s.									
Inflores- cence						63	20.2	22.2	17.2
						WM <i>v.</i> WS, $P < 0.05$; C <i>v.</i> WS, and WM <i>v.</i> C, n.s.			

* Comparisons at time of harvest are at least significant at the level $P < 0.01$, unless otherwise stated. n.s.: not significant.

been due to more favourable conditions for recovery after re-watering in experiment 2, and to the delay of 4 hr in commencing harvest 4 in this experiment. The relative water content values for experiment 1 suggest that recovery was most rapid for the laminae and least rapid for the stem.

TABLE 3
EFFICIENCY AND RATES OF TRANSPIRATION

Transpiration	Harvest	Experiment 1				Experiment 2			
		Control	Moderate Wilting	Severe Wilting	P*	Control	Moderate Wilting	Severe Wilting	P*
Efficiency (g dry matter/l. water transpired)	2-3	3.37	4.10	4.05	n.s.	4.76	6.45	6.67	<0.001
	4-5	3.49	3.03	3.04	n.s.	4.60	4.10	4.31	n.s.
	5-6	3.06	3.31	2.92	n.s.	6.33	6.93	7.01	n.s.
Rate per unit lamina dry weight (g water used/g lamina dry wt./day)	2-3	69.4	47.9	33.1	<0.001	45.3	28.5	19.4	<0.001
	4-5	58.9	65.7	70.5	<0.05	29.2	31.3	30.6	n.s.
	5-6	58.4	65.2	66.0	n.s.	20.6	23.2	24.0	<0.05
Rate per unit leaf area (g water used/ sq. dm./day)	2-4	26.6	19.5	12.9	<0.001				
	4-5	22.0	25.0	26.7	n.s.				
	5-6	23.4	27.0	26.2	n.s.				

* Comparison of control and severe wilting only. n.s.: not significant.

(e) Water Usage

Transpiration data are presented for both experiments in Table 3 as the efficiency of transpiration and the transpiration rate per unit lamina dry weight and per unit leaf area.

Both WM and WS cause a marked increase in values for efficiency of transpiration for interval 2-3, but the severity of wilting has little effect on the values. Upon recovery from wilting the efficiency of transpiration does not differ significantly between treatments.

Transpiration rate per unit dry weight of lamina is similar in trend for the two experiments, although much more water was transpired in experiment 1 than in experiment 2, no doubt because of the higher humidity and the lower temperature in experiment 2. There is a highly significant depression in the rate during wilting, the values being about 65 per cent. of control for WM and 45 per cent. for WS in both experiments. Upon returning to field capacity, the transpiration rates are approximately the same for both experiments, although the values are slightly higher for the wilting treatments than for control, and even attain a significant difference at one stage in each experiment.

Leaf areas are only available throughout growth for experiment 1, and therefore transpiration rate on an area basis can be determined only for that experiment, but, as the values closely parallel those on a weight basis, it may be inferred that the trends would be the same in the second experiment. The depression due to wilting is highly significant and of a similar order to that on a weight basis, and transpiration rate upon recovery is similar to control, although apparently slightly higher.

IV. DISCUSSION

Every effort was made in designing the experiments to ensure that a high plane of nutrition would be maintained and that treatment would not be confounded with nutrient status. The soil chosen had a high available phosphorus and nitrogen content and the nutrient addition before the first harvest was at a high rate in each experiment. To be doubly sure that the treatment effects observed in experiment 1 were not contributed to by nutrient shortage, the additional dressings of phosphorus and nitrogen were made on two occasions after wilting in experiment 2. However, the plants of the two experiments show a remarkably similar pattern of response to the wilting treatments. It is apparent, therefore, that experiment 1 was not short of nutrient and that the effects may reasonably be attributed to the water treatments.

(a) Ontogenetic Drifts and Moisture Stress

The work of Goodall (1945, 1946) gives a basis for comparison with the results of the work reported here, because of the fact that a similar eight-leaf stage of development was chosen for imposing the wilting treatments. Goodall studied assimilation, respiration, and translocation by following during 24 hr the dry weight changes both in the intact plant and in the separated organs. The values noted here for relative growth rates, net assimilation rates, and leaf weight ratios are comparable to Goodall's values for the spring or autumn, but are not as high as his summer values nor as low as those for winter. Goodall found that translocation normally proceeded from the lower leaves to the upper leaves, the stem, and the root. Using his estimates for summer and winter, it may be deduced that the dry matter translocated to the stem and the root would be partitioned in nearly equal proportions for spring or autumn grown plants. Goodall also found that dry weight increase in the stem corresponds to the amount of dry matter translocated to it, assimilation and respiration being approximately equal.

In the present experiments, it has been observed that the dry weight of the whole plant at the final harvest was depressed by the water shortage. This net response was the resultant of two differing trends, that during and that subsequent to wilting, but there was no indication that the second trend was complete at the final harvest. During wilting, the relative growth rates and the net assimilation rates were markedly depressed, but in the first harvest interval after wilting values did not differ from control, whilst in the next, and final, harvest interval the values for the wilted plants were significantly greater than those for control. The response of WM plants was intermediate to that

of control and WS. The difference in response before and after wilting is also apparent from the trends in the weight ratios of laminae and stem. During wilting, stem weight ratio increased and lamina weight ratio decreased; subsequent to wilting the relationship was the reverse of this.

Petrie and Arthur (1943) obtained an insignificant depression in dry weight during the water shortage and a higher growth rate considerably after re-watering in their early temporary drought treatment. However, this treatment is not strictly comparable with the present wilting experiments because it represents a reduction of water levels from well above moisture equivalent to slightly below moisture equivalent, and certainly to a point quite high in the available range. Their late temporary drought is more comparable to the present treatments, for soil moisture was then reduced below the permanent wilting percentage. This treatment was applied at a much later stage of growth, but drought caused a significant depression in dry weight followed by a higher growth rate subsequent to re-watering.

The work reported here shows that all plant parts increased in dry matter content during the interval of wilting, so that no one part can be said to have developed to the entire suppression of another. Translocation between parts, however, did occur. The relative growth rates for laminae and stem during that interval are as follows (in g/100 g/day):

Experiment:		1	2	1	2	1	2
		Control		Moderate Wilting		Severe Wilting	
Plant Part	Laminae:	9.15	8.85	6.12	6.59	3.74	4.28
	Stem:	13.64	12.64	13.82	11.88	9.89	9.07

With WS the values for laminae were reduced by approximately 50 per cent., and with WM by approximately 30 per cent. Similar comparisons for the stem are 25 per cent. and almost nil. Thus during both wilting treatments stem development was not retarded to the same extent as that of the laminae. Stem development would be almost entirely at the expense of the laminae (Goodall 1946); indeed it is probable that the stem would assimilate less dry matter during wilting than it used in respiration. Translocation from the laminae to the stem must therefore have been enhanced in the wilted plants when compared with the controls. The effect of treatment on weight ratios during wilting expresses these facts in another way and these effects were established with significance.

The changed relationship between lamina and stem weight ratios upon re-watering does not necessarily imply that translocation back to the laminae from the stem occurred, but could be interpreted that the dry matter assimilated by the laminae was retained by them in greater proportion upon re-watering. Although data for relative growth rates of plant parts are not presented, *R* values for laminae rose above control before those of the stem, especially in experiment 1, and this is an indication of enhanced development of the laminae. The effects of treatment on dry weight accumulation in the whole plant and its

parts, as expressed in growth rates, weight ratios, and translocation between parts may be interpreted as a tendency towards senescence during wilting and a return to a more juvenile condition upon re-watering, for with advancing age, in the normal plant, it is usual for growth rates to fall, stem weight ratios to rise, and lamina weight ratios to fall. Morton and Watson (1948) found for leaf development in sugar beet that transition from high to low water regime hastened the appearance of senescence, whilst the reverse transition delayed it. They also found a very considerable increase in the net assimilation rate of plants receiving high levels of nitrogen when changed from low to high water supply.

The assumption has too often been made that plant growth is unaffected by soil moisture stress until the permanent wilting percentage is attained. It has already been emphasised that the soil water in these experiments was at no time below the permanent wilting percentage; indeed, soil water in WM was well above permanent wilting percentage for most of the period during which water was withheld. Even in the latter treatment, however, there was a highly significant depression in growth, and it is surprising that the depression was so great as to be half that of WS, especially when it be remembered that the plants were re-watered during this period. Consideration of this fact suggests that the depression of growth developed gradually in both WM and WS and could have commenced at a point high in the available range of water supply. For if the effect developed suddenly towards the lower soil moisture levels of WM, then the growth depression in the case of WS should be many times greater than was found for WM. In this connection, reference may be made to the findings of Wadleigh and Gauch (1948) concerning the rate of leaf elongation in cotton as affected by the intensity of the total soil moisture stress. They found that low moisture stresses of from one to three atmospheres were without observable effect on leaf elongation, but at higher values of stress elongation was progressively reduced. They derived values close to 15 atm, or the permanent wilting percentage, for the cessation of leaf growth.

It has been inferred from the present study that soil moisture can be limiting to plant growth at a point relatively high in the available range. The actual impairment of growth, however, is the result of a fall in the levels of tissue moisture, and these are affected by both soil moisture and atmospheric humidity. In these experiments and especially in the second, humidity was maintained at high levels in order that tissue water might fluctuate as nearly as possible in response to soil treatment alone. Marked changes in water content did occur in response to treatment, as is obvious from the data, but no precise inferences concerning their nature at particular stages of wilting are possible. The particular value of obtaining such information is apparent from references in the literature. Several workers have found water levels in the leaves of various species to be important for active carbon assimilation. Dastur (1925) found a linear relationship between leaf water content and assimilation of carbon dioxide, Dastur and Desai (1933) found that carbon dioxide assimilation by leaves was related more to water content than to chlorophyll content, Melville (1937) found that gain in dry weight in seedling tomatoes increased

as water content increased, and Goodall (1946) found that carbon assimilation was much less in wilted tomato leaves than in turgid ones.

Tissue water levels have also been suggested as an important factor in the active functioning of the cell and in protein synthesis. This is not only important during wilting but also during the recovery period. Petrie (1943) has summarized certain aspects of this relation and has claimed that it is certainly possible, although not definitely certain, that water content affects the velocity constants of synthesis and hydrolysis of protein. Robinson and Brown (1952) found a close connection between the growth, protein content, and enzyme activities of individual cells of the root tip of the broad bean. They assessed growth of the cells in terms of the water content, which was taken as a measure of volume. They found that enzyme activity changed intimately with the level of protein, but that this activity was not being determined simply by the level of protein. They claimed that their data suggested that protein may have had an indirect effect on growth through the determination of the level of enzyme activity. Robinson and Brown do not suggest that water content was a determinant of these effects, but their effects on growth were actually effects on water content so that water content as such may be relevant to the results obtained. It has been suggested by Barer, Ross, and Tkaczyk (1953) that many types of cellular activity are accompanied by an increase in water content of cells or of portions of cells in a number of organisms.

As water content appears to be closely related to active carbon assimilation in the leaves, to the synthesis of protein, and to active metabolism in the cell, it would seem to be of the utmost importance to determine just what tissue water levels are required to maintain these processes at optimal rates.

(b) Water Usage

The values for rates of transpiration per unit of leaf tissue show marked treatment differences during wilting. These values are means of values that were initially high and equal for all treatments, but were later low and dissimilar for treatments; for example, 85 per cent. of the water transpired by WS had been lost when WM was re-watered for the first time. There were thus very different transpiration patterns for the two wilting treatments. The ratio of dry matter produced to water used was virtually the same for WM and WS during wilting, which means that the major contribution to such a result must have been derived from similar parts of the drying curve. This would be from the early stages of wilting. This is further evidence that active dry matter production occurred only when transpiration was active and leaf water contents were high.

The high values for efficiency of transpiration during wilting may arise from diurnal fluctuations in turgidity, and therefore in all probability of growth, during the progress of the wilting treatments. Full turgidity during wilting could be expected only in the early morning, when transpiration would be low. Conditions might then be favourable for active carbon assimilation, but not later in the day. However, sufficient has been said to show that the characteristics of growth under water shortage are different from those under full water

supply and it must be remembered that the increment in dry weight is slight. The apparently greater efficiency would appear to be of doubtful value.

Kramer (1950) claimed that, after re-watering sunflower and tomato plants following a period of water shortage, the transpiration rates were considerably reduced. After 4 days they were 70 or 80 per cent. of the normal. Unfortunately, differences in leaf area or leaf weight developing between wilted plants and controls during treatment were not mentioned, so that it is difficult to know whether rates per unit area or per unit weight were significantly affected or not. In the data now presented, plants of the wilted series transpired much less total water upon re-watering, but the effect was due to a retarded development of leaf tissue. Transpiration per unit lamina area and dry weight for the wilted plants were either the same as or higher than the controls. The data more nearly parallel Kramer's results with detopped tomato plants connected to the vacuum pump, where water flow under suction rose above controls within 4 days.

Loustalot (1945) found for pecans, and Schneider and Childers (1941) for apples, that the rates of transpiration of selected leaves reached normal several days after re-watering from the wilted condition, and values then rose slightly above normal. Their observations were made daily and on an area basis. The temporary reduction of transpiration rate that was observed for these individual leaves may well have been compensated for by younger leaves on the plant, but at best it had only a transient influence on the water economy of the plant after re-watering. In the data now presented, values of transpiration on a unit leaf basis appear to have almost immediately returned to normal or slightly above. It can be concluded that the period of wilting did not effect any economy in subsequent water usage per unit of leaf weight or leaf area. This is contrary to the commonly held view that wilting plants "trains" them to economize in their use of water.

V. ACKNOWLEDGMENTS

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RESPONSE OF TOMATO PLANT TO WATER SHORTAGE. I



Plants of the smallest and largest size groups in both experiments at the final stage of wilting, when re-watering was commenced. The smallest size groups are at the top of the plate, and plants of experiment 1 are on the left.

Figs. 1 and 2.—Plants of experiment 1. Photographs taken indoors. From left to right, treatments are control, severe wilting, and moderate wilting respectively.

Figs. 3 and 4.—Plants of experiment 2. Photographs taken in bright sunshine. From left to right treatments are control, moderate wilting, and severe wilting respectively.

THE RESPONSE OF THE YOUNG TOMATO PLANT TO A BRIEF PERIOD OF WATER SHORTAGE

II. THE INDIVIDUAL LEAVES

By C. T. GATES*

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Summary

The dry weight and water contents data are considered for the individual laminae and petioles of the first eight leaves of tomatoes subjected to a brief period of wilting after they had developed eight easily manipulable leaves. The aim was to obtain a better understanding of the response previously described for the whole plant, by studying the growth of organs which were at various stages of development when treatments were imposed.

It was found that the rise in stem weight ratio and fall in lamina weight ratio during wilting occurred because translocation continued to the stem, but was impaired to the upper laminae. Upon re-watering, the normal course of translocation appeared to be readily resumed.

The decreased growth rates of the laminae during wilting and the resurgence of their active growth upon recovery can now be seen to derive principally from the response of the younger laminae and the younger tissues of the more mature laminae.

Both the growth data and the water content data of the individual leaves show that it is the younger portions of the total leaves that are important centres for the development of the whole plant response, a response which may be described as a senescent decline in growth during wilting and the development of a physiologically younger condition upon recovery.

I. INTRODUCTION

The data for the plant as a whole and its major parts have been presented in Part I of this series (Gates 1955). The response to water shortage involved two differing trends, that during and that subsequent to wilting. Water stress caused a decline in growth whilst re-watering led to development reminiscent of a physiologically younger condition than that of the controls at that time. In order to obtain a better understanding of the internal factors involved in this response it seemed wise to study the growth of the individual laminae and petioles, for they were in various stages of development when the treatments were imposed. The data for dry weight and water content are considered in the present paper.

Goodall (1945, 1946) studied changes in dry weight of individual leaves and translocation between leaves over 24 hr periods for tomato plants at the eight-leaf stage, and compared trends throughout the course of a year. His results represent the response of a plant grown to a predetermined stage under

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a range of seasonal conditions, and therefore give a useful basis for comparison with the control series of the data now presented, where treatment was commenced at a similar eight-leaf stage.

II. EXPERIMENTAL

Cultural details and the procedure in harvesting have been described for experiments 1 and 2 in an earlier paper (Gates 1955). For convenience, it may be repeated that the laminar fractions comprised the blades of the leaflets of each leaf, separated at the junction with the petiolules; and the petiolar fractions included the main axis and petiolules.

Areas for the individual leaves were obtained differently in the two experiments. In experiment 1, the individual estimates made in assessing the areas of the whole plant were used. These were based on leaf-area standards set up by Williams (1954). In experiment 2, a direct photographic method was used. The leaves were photographed without damaging the plant, using a wide angle lens at fixed focal length. In photographing the leaf a padded board was placed beneath it and a sheet of celluloid, ruled into a sq. cm. grid, was placed over it. The base board carried a rigid, recurved arm to which the camera was attached, thus ensuring the fixed focal length. On the average, photographs took 15 sec each to take. The negatives obtained bore the sq. cm. grid superimposed upon them, and the leaf areas were assessed by projecting them in a darkened room and counting the squares and portions of squares.

Improved estimates of lamina dry weight data were derived for each experiment using leaf area measurements prior to harvest. The method of McIntyre and Williams (1949, p.343) was used, except that the procedure for experiment 1 was modified as follows. For each leaf position, the mean weights of leaves for each treatment at each harvest were adjusted for variation of the mean leaf area from the general mean leaf area at the time of the second harvest, which preceded the application of treatments. The regression coefficients of leaf weight at subsequent harvest on initial leaf area varied considerably, and a smoothing procedure was adopted equivalent to fitting a regression surface to these regression coefficients as a function of leaf position and harvest. There was no evidence of treatment differences in regression at particular positions at any harvest. Logarithmic transformations of the basic data were used in experiment 1, but not in experiment 2.

III. RESULTS

As in Part I of this series (Gates 1955), treatments will be referred to as: Control (C): frequent watering to field capacity; WM: brief period of moderate wilting; WS: brief period of severe wilting.

(a) Experiment 1

(i) Primary Data

Table 1 presents the logarithms of mean dry weights for the individual laminae (improved estimates), the cotyledons, and the rest of the laminae grouped together. Figure 1 shows trends in absolute lamina dry weight.

The early leaves of the tomato tend to emerge in pairs, and later develop the phyllotaxis characteristic of the species. Leaves 1 and 2 are almost opposite, leaves 3 and 4 are less obviously so, and after that the tendency to pairing is rapidly lost. These characteristics are reflected in the growth curves for the individual laminae of the control plants (Fig. 1), for the form and slope of the curves change markedly between laminae 2 and 3, and between laminae 4 and

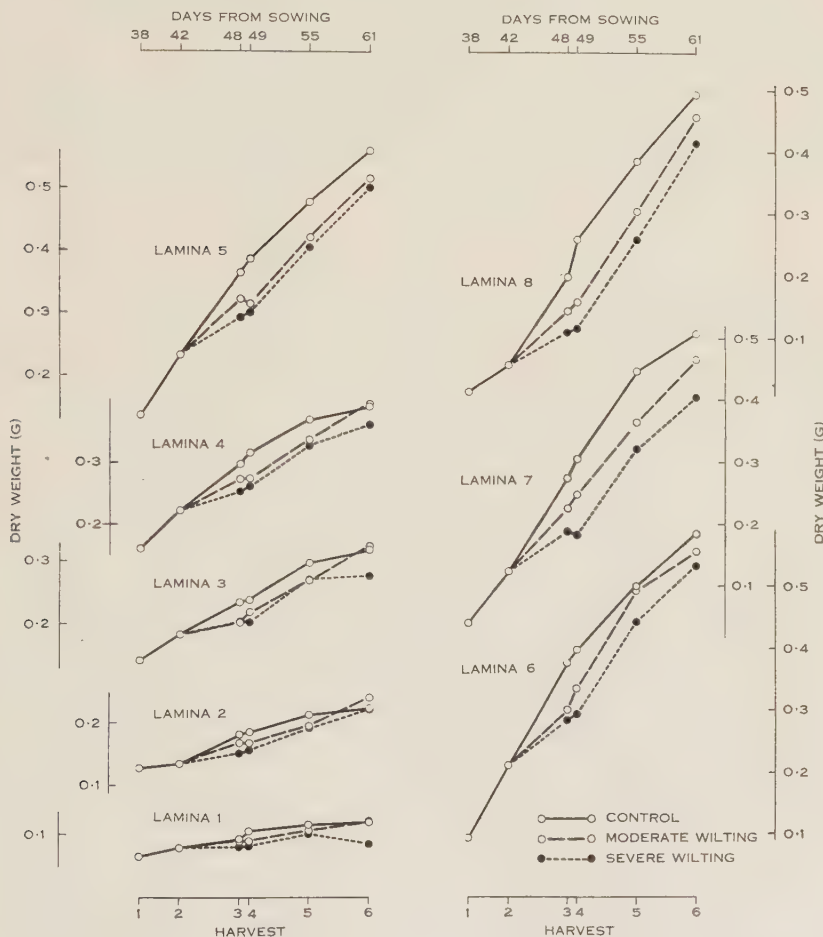


Fig. 1.—Dry weights of the laminae of the first eight leaves, experiment 1.

5. The changes in form are more pronounced above lamina 6 and it will be noted that there are fairly definite inflections in the curves of laminae 7 and 8. The stepwise nature of the changes in growth rate with successive laminae is well shown by their relative growth rates for the period of the experiment, as is apparent from the data of Table 2.

Treatment effects are not apparent in the cotyledons, but they occur in lamina 1 and increase in magnitude for each lamina in passing up the plant, as is to be expected considering the stage of growth of each leaf when treatment was imposed. Water shortage retarded the development of all laminae.

In the moderately wilted plants at the conclusion of wilting, all laminae differ from control but the lower four laminae show few significant differences.

TABLE 1
LOG DRY WEIGHT (CG) OF COTYLEDONS AND LAMINAE:† EXPERIMENT I

Plant Part	Harvest 1:Day 38				Harvest 2:Day 42			
	C	WM	WS	C v. WS	C	WM	WS	C v. WS
Cotyledons	<i>0.4232</i>				<i>0.4487</i>			
Lamina 1	<i>0.8075</i>				<i>0.8943</i>			
2	<i>1.1045</i>				<i>1.1290</i>			
3	<i>1.1430</i>				<i>1.2598</i>			
4	<i>1.2039</i>				<i>1.3453</i>			
5	<i>1.1277</i>				<i>1.3659</i>			
6	<i>0.9731</i>				<i>1.3251</i>			
7	<i>0.6222</i>				<i>1.0911</i>			
8	<i>0.1303</i>				<i>0.7548</i>			
Rest	—				<i>0.6191</i>			
	Harvest 3:Day 48				Harvest 4:Day 49			
	C	WM	WS	C v. WS	C	WM	WS	C v. WS
Cotyledons	<i>0.4757</i>	<i>0.5145</i>	<i>0.4082</i>	—	<i>0.5328</i>	<i>0.4564</i>	<i>0.4698</i>	—
Lamina 1	<i>0.9627</i>	<i>0.9472</i>	<i>0.9009</i>	*	<i>1.0197</i>	<i>0.9512</i>	<i>0.9137</i>	***
2	<i>1.2564</i>	<i>1.2223</i>	<i>1.1778</i>	**	<i>1.2637</i>	<i>1.2225</i>	<i>1.1937</i>	**
3	<i>1.3676</i>	<i>1.3051</i>	<i>1.3054</i>	*	<i>1.3747</i>	<i>1.3380</i>	<i>1.3043</i>	**
4	<i>1.4702</i>	<i>1.4335</i>	<i>1.4018</i>	—	<i>1.4978</i>	<i>1.4346</i>	<i>1.4150</i>	***
5	<i>1.5619</i>	<i>1.5071</i>	<i>1.4670</i>	***	<i>1.5874</i>	<i>1.4976</i>	<i>1.4782</i>	***
6	<i>1.5759</i>	<i>1.4786</i>	<i>1.4528</i>	***	<i>1.5998</i>	<i>1.5239</i>	<i>1.4664</i>	***
7	<i>1.4382</i>	<i>1.3536</i>	<i>1.2749</i>	***	<i>1.4318</i>	<i>1.3933</i>	<i>1.2612</i>	***
8	<i>1.2975</i>	<i>1.1578</i>	<i>1.0398</i>	***	<i>1.4115</i>	<i>1.1967</i>	<i>1.0323</i>	***
Rest	<i>1.3672</i>	<i>1.1011</i>	<i>0.8543</i>	***	<i>1.4196</i>	<i>1.2146</i>	<i>0.8609</i>	***
	Harvest 5:Day 55				Harvest 6:Day 61			
	C	WM	WS	C v. WS	C	WM	WS	C v. WS
Cotyledons	<i>0.5682</i>	<i>0.5403</i>	<i>0.4346</i>	*	<i>0.5527</i>	<i>0.5809</i>	<i>0.5635</i>	—
Lamina 1	<i>1.0604</i>	<i>1.0233</i>	<i>0.9986</i>	*	<i>1.0746</i>	<i>1.0809</i>	<i>0.9267</i>	***
2	<i>1.3250</i>	<i>1.2879</i>	<i>1.2799</i>	—	<i>1.3462</i>	<i>1.3787</i>	<i>1.3446</i>	—
3	<i>1.4722</i>	<i>1.4274</i>	<i>1.4301</i>	—	<i>1.5018</i>	<i>1.5099</i>	<i>1.4394</i>	*
4	<i>1.5641</i>	<i>1.5240</i>	<i>1.5130</i>	*	<i>1.5891</i>	<i>1.5929</i>	<i>1.5540</i>	—
5	<i>1.6789</i>	<i>1.6227</i>	<i>1.6073</i>	**	<i>1.7474</i>	<i>1.7113</i>	<i>1.7001</i>	—
6	<i>1.6986</i>	<i>1.6921</i>	<i>1.6461</i>	*	<i>1.7668</i>	<i>1.7443</i>	<i>1.7249</i>	—
7	<i>1.6508</i>	<i>1.5605</i>	<i>1.5078</i>	***	<i>1.7073</i>	<i>1.6695</i>	<i>1.6083</i>	***
8	<i>1.5842</i>	<i>1.4820</i>	<i>1.4115</i>	***	<i>1.6937</i>	<i>1.6596</i>	<i>1.6187</i>	**
Rest	<i>1.8565</i>	<i>1.7103</i>	<i>1.6295</i>	***	<i>2.1132</i>	<i>2.0231</i>	<i>2.0015</i>	**

† Improved estimates of experimental values, excepting that figures in italics are experimental values.

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

whereas the upper four differ quite markedly and with high significance. At harvest 4, on the day following watering to field capacity, all laminae (except 5) appear to have risen in dry weight, but significant differences for this short interval cannot be established.

Comparison of the severely wilted plants with control at the conclusion of wilting shows that all laminae had failed to develop to the control level, the differences being significant (except for lamina 4) and in the upper laminae highly significant. Upon re-watering, it appears that dry weight tended to increase in the laminae generally, but once again the significances for the 1-day interval to harvest 4 cannot be established.

Table 3 presents logarithms of the dry weights of the petioles of the first eight leaves. The logarithms are presented because direct comparison with Table 1 is then more easily made on a proportional basis. In the control series the trend of development of each petiole is very similar to that of the corresponding lamina, though petiole development is always delayed by comparison with the lamina. This similarity is apparent from the successive relative growth rates for the period of the experiment (Table 2).

TABLE 2
RELATIVE GROWTH RATES FOR THE PERIOD OF EXPERIMENT I (G/100 G/DAY)

Leaf	Lamina	Petiole	Leaf	Lamina	Petiole
1	2.67	3.10	5	6.20	8.94
2	2.42	2.82	6	7.95	11.87
3	3.59	4.77	7	10.86	15.64
4	3.86	5.85	8	15.65	—

Comparison of the moderately wilted series with control at harvest 3 shows that the dry weights of petioles 1, 2, and 4 rise above control (the significances being n.s., $P < 0.001$, and $P < 0.001$ respectively), that petioles 3 and 5 do not differ significantly from control and petioles 6, 7, and 8 fail to develop to control level (P being < 0.001 , < 0.05 , and < 0.01 , respectively). The trend of response is thus different in the petioles from that in the laminae; the petioles show higher and the laminae lower values than control in the older leaves. At harvest 4, the dry weights of petioles 1, 2, 4, and 5 appear to have fallen, but those of petioles 6, 7, and 8 appear to have risen. However, significances cannot be established for differences over this interval.

Comparison of dry weights of petioles of the severely wilted series with those of control at harvest 3 shows that petioles 1-4 do not differ significantly from, but tend to be above control at the conclusion of wilting, whereas those of petioles 5, 6, 7, and 8 are significantly below control. This contrasts with the laminae where all values were significantly less than control in the severely wilted series. Upon re-watering, values at harvest 4 appear to have risen for the petioles of the four older leaves but to have fallen for the petioles of the four younger. Significances for these slight differences cannot be established, but they tend to be the reverse of those for the moderately wilted series.

Upon recovery from moderate wilting, the four lower laminae regain control levels by the final harvest but the four upper do not. The petioles behave similarly. In the severely wilted plants neither laminae nor petioles regain

control level after re-watering, but for the majority of the leaves the gap closes rather than widens, and the significance of comparisons C *v.* WS is less at harvest 6 than at harvest 5.

(ii) *Relative Growth Rates*

Figure 2 presents, in oblique parallel projection, the relative growth rates R , of the eight laminae for successive harvest intervals.

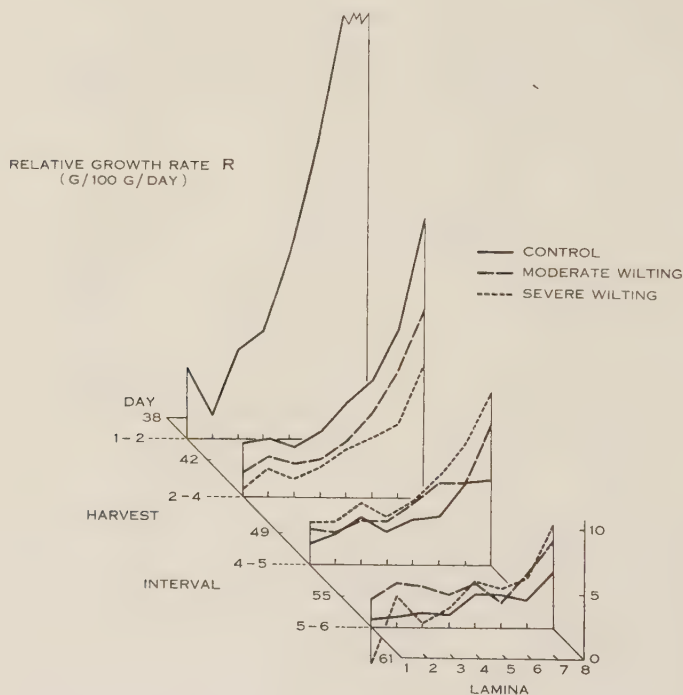


Fig. 2.—Relative growth rates, R , of the laminae of the first eight leaves, experiment 1. Oblique parallel projection.

For each interval, relative growth rates of the control series increase fairly consistently with leaf position in passing up the plant, but all rates fall with time; values for the upper laminae falling more sharply than those for the lower. The response to wilting is a modification of this pattern. During wilting (interval 2-4), R is depressed with high significance for all laminae, the moderately wilted being intermediate to the control and severely wilted. During the interval following wilting (interval 4-5) the relationship to control is the inverse of that during wilting, the severe wilt treatment now having the highest R values for all laminae. Differences are significant for only the three upper laminae when considered individually, but the comparison WS *v.* C for the total laminae is significant at the level $P < 0.01$. During the final interval, values for wilted plants remain higher than control but significance can only be

established for lamina 8. The depressing effect of wilting on *R* values is greater in an absolute sense for the upper laminae, and the upper laminae exhibit the more significant and longer continued response in recovery.

TABLE 3
LOG DRY WEIGHT (CG) OF PETIOLES: EXPERIMENT 1

Plant Part	Harvest 1:Day 38				Harvest 2:Day 42			
	C	WM	WS	C v. WS	C	WM	WS	C v. WS
Petiole 1	0.4232				0.5428			
2	0.7110				0.7896			
3	0.7259				0.9294			
4	0.7210				0.9445			
5	0.5575				0.9274			
6	0.3201				0.8363			
7	1.8808				0.5302			
8	—				0.0569			
Rest	—				—			
	Harvest 3:Day 48				Harvest 4:Day 49			
	C	WM	WS	C v. WS	C	WM	WS	C v. WS
Petiole 1	0.6294	0.6830	0.6335	—	0.7118	0.6355	0.6665	—
2	0.8785	0.9736	0.9042	—	0.9430	0.9304	0.9405	—
3	1.0682	1.0399	1.0531	—	1.0842	1.0885	1.0526	—
4	1.1096	1.1596	1.1152	—	1.1914	1.1370	1.1464	*
5	1.2044	1.1979	1.1405	**	1.2584	1.1835	1.1364	***
6	1.2204	1.1319	1.0976	***	1.2342	1.1869	1.0671	***
7	1.0645	0.9400	0.8370	**	1.0816	1.0107	0.7672	***
8	0.8525	0.7050	0.4914	***	0.9685	0.7505	0.4314	***
Rest	0.7980	0.4786	0.1644	***	0.8615	0.6335	0.1931	***
	Harvest 5:Day 55				Harvest 6:Day 61			
	C	WM	WS	C v. WS	C	WM	WS	C v. WS
Petiole 1	0.7589	0.7007	0.6803	—	0.7324	0.7160	0.6857	—
2	1.0338	0.9619	0.9425	*	0.9930	1.0692	0.9877	—
3	1.1818	1.1580	1.1186	—	1.2025	1.2277	1.1314	*
4	1.2664	1.2217	1.2125	*	1.3054	1.3065	1.2471	*
5	1.3691	1.3054	1.2650	***	1.4501	1.4259	1.3791	***
6	1.3691	1.3602	1.3100	***	1.5055	1.4582	1.3709	**
7	1.3367	1.2153	1.1459	***	1.4436	1.3836	1.2465	***
8	1.3009	1.1647	1.0378	***	1.4713	1.4048	1.2898	***
Rest	1.4824	1.2792	1.1513	***	1.8420	1.6994	1.6476	***

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(iii) Water Content

Figures 3 and 4 present the data for relative water and absolute water for the laminae and petioles of the first eight leaves and for the stem on each harvest occasion. The scales for the absolute data and for the relative data have been adjusted, for each figure, so that the mean of the values for each plant part is

the same. Improved estimates of the experimentally determined lamina water data are presented, these being derived by the same procedure as the improved estimates of lamina dry weight, except that a freehand curve was drawn for the relationship between leaf position and harvest. As significances could then not be determined, they were estimated from the experimental values.

In the controls, the relative water contents of the petioles especially, but also of the laminae, change in passing up the plant. This applies to all harvests but especially the earlier ones. In harvests 1 and 2, the change is an overall fall in values but with higher values for the medianly situated petioles. At

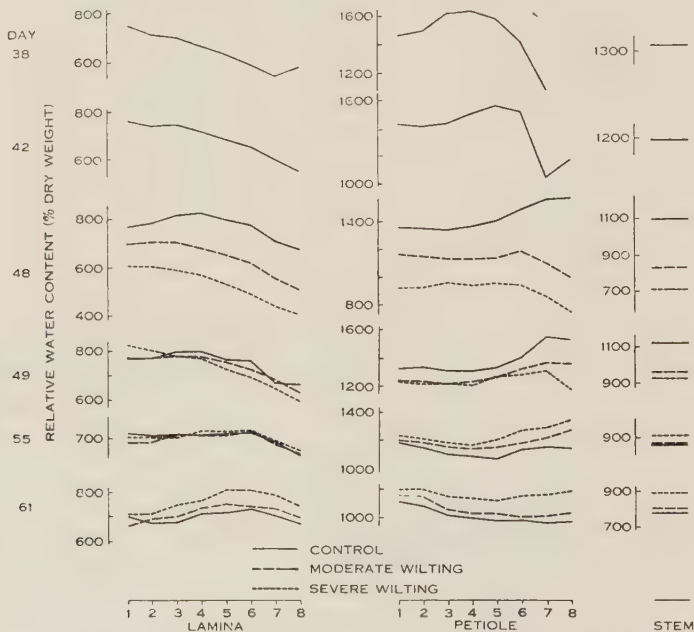


Fig. 3.—Relative water content of the laminae and petioles of the first eight leaves and of the stem, experiment 1.

harvests 3 and 4 this trend is continued for, whilst the values for the petioles rise in the graph, they fall again in the “rest of petioles” to 1325 and 1296 per cent. respectively, which is lower than for the other petioles. This trend is also evident at harvests 3 and 4 in the laminae, for values for the median laminae are high whilst those for the “rest of laminae” are lower than for any other lamina, being 635 and 614 per cent. There is thus in these earlier harvests an overall decline in the values for relative water in passing up the plant, but there is also a marked tendency for the highest values to be developed in the medianly situated leaves. Portyanko (1948) and Gupalo (1949) have published data and have referred to the literature in support of the claim that there is a negative gradient in the relative water contents of the leaves of a plant in passing up the plant. The data presented here show that this is not always the case.

WM and WS cause changes in relative and absolute water content both during wilting and subsequent to wilting. The fall in values for relative water content of both laminae and petioles during wilting was referred to in Part I. It is here seen to occur in all leaves, though the effect is greater in the upper leaves, including the "rest of leaves" where the values drop still further for both fractions. This result is of interest in view of the generally held opinion that in passing up the plant, prolonged wilting reverses the normal gradient of relative water content from negative to positive (Maximov 1929, p.337, 1941, p.306; Crafts, Currier, and Stocking 1949, p.173).

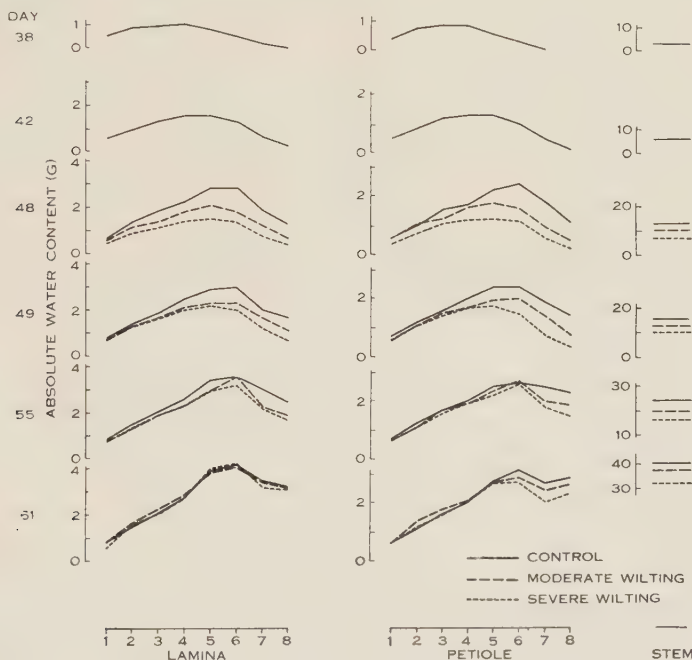


Fig. 4.—Absolute water content of the laminae and petioles of the first eight leaves and of the stem, experiment 1.

Recovery upon re-watering is rapid. One day after re-watering (day 49) there is no significant treatment difference in relative water content for any of the laminae, and values for the petioles have almost regained control level. At one week after re-watering (harvest 5), there are no significant differences in relative water content for any laminae, although in the petioles values for WS are significantly higher than for control for all except petiole 1. At the final harvest, the values for all laminae, except 1 and 2, and for all petioles are significantly higher in WS than in the control. They are also higher in WM, although not significantly so.

For laminae and petioles in the control series the absolute water contents show a similar trend in passing up the plant. Treatment effects on absolute water during wilting are also similar for laminae and petioles and are largest in the upper leaves. Upon re-watering, both WM and WS tend to overtake

control, and all the eight laminae attain to control values by the final harvest. The petioles of the upper leaves, on the other hand, do not overtake control by the final harvest, neither do the values for the stem, as already noted in Part I. Significances of comparisons C *v.* WS for each harvest are given in Table 4 for the absolute water content of all parts.

TABLE 4
SIGNIFICANCE OF ABSOLUTE WATER CONTENT COMPARISONS: EXPERIMENT 1
Comparisons C *v.* WS at successive harvests

Plant Part	Harvest Day				Plant Part	Harvest Day			
	48	49	55	61		48	49	55	61
Cotyledons	***		*		Stem	***	***	***	***
Lamina 1	*	*			Petiole 1	*			
2	***		*		2	***			
3	***	*			3	***	*		
4	***	***			4	***	**		
5	***	***	*		5	***	***		
6	***	***			6	***	***		
7	***	***	**		7	***	**	**	**
8	***	***	***		8	***	***	***	**
Rest	***	***	***	*	Rest	***	***	***	*

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(b) Experiment 2

(i) Primary Data

Table 5 presents dry weights for the laminae and petioles of leaves 2 and 6 and for lower and upper leaf groups. The second and sixth leaves have been selected as representative of these groups.

The 4 hours' delay in removing the plants to the laboratory and commencing harvest 4 has caused some of the values at harvest 4 to appear to be in error. This has occurred because sampling was performed at a different point in the diurnal rhythm of change in dry weight. Apart from this harvest, the control series shows a regular increase in dry matter, but at harvest 4 the values are unduly high in the lower laminae and petioles. Goodall has shown for the tomato at the eight-leaf stage that the lower leaves assimilate more dry matter than they use in growth, and that translocation proceeds from the lower laminae to the upper laminae, to the stem and to the root. It is therefore likely that the delay in harvest would lead to the assimilation of dry matter which would not have been translocated from the lower laminae. Dry weight values of the lower laminae would therefore be unduly high. This fact tends to complicate the data for the controls at this harvest and to make interpretation difficult. WM appears to have also shared in the effect, but WS does not, perhaps because recovery from wilting was not then complete.

At harvest 3 for WM the dry weight of laminae 1-4 is less than control, but the petioles of this same group of leaves are slightly heavier than control, although the difference is not statistically significant. Similarly, lamina 2 is

TABLE 5
DRY WEIGHTS (G) OF LAMINAE AND PETIOLES*: EXPERIMENT 2

Plant Part	Harvest	Day	Control (C)	Moderate Wilt (WM)	Severe Wilt (WS)	Plant Part	Control (C)	Moderate Wilt (WM)	Severe Wilt (WS)
Lamina 2 (improved estimates)	1	36	0.2080			Lamina 6 (improved estimates)	0.1623		
	2	40	0.2245				0.2911		
	3	48	0.3163	0.2868	0.2604		0.6281	0.5128	0.4257
	4	49	0.3308	0.2810	0.2769		0.6772	0.6269	0.4722
	5	56	0.3496	0.3439	0.3176		0.7524	0.6509	0.6268
	6	63	0.3671	0.3682	0.3540		0.9230	0.7868	0.7906
			C v. WS: $P < 0.05$ at harvest 5; n.s. at harvest 6.						
Laminae 1-4	3	48	1.4774	1.4201	1.1988	Laminae 5 and above	2.4435	1.8483	1.5195
	4	49	1.6815	1.5429	1.3342		2.7117	2.1378	1.7015
	5	56	1.6398	1.6238	1.5254		3.8517	3.0596	2.5665
	6	63	1.9399	1.8734	1.7996		5.2341	4.6203	4.2692
			C v. WS: n.s. at harvest 5; $P < 0.05$ at harvest 6.						
Petiole 2	1	36	0.0753			Petiole 6	0.0391		
	2	40	0.0899				0.1101		
	3	48	0.1396	0.1582	0.1292		0.3485	0.2656	0.2095
	4	49	0.1597	0.1421	0.1405		0.3596	0.3301	0.2160
	5	56	0.1579	0.1674	0.1625		0.5121	0.3714	0.3315
	6	63	0.1684	0.1712	0.1700		0.5503	0.4496	0.4105
			C v. WS: n.s. at harvests 3-6.						
Petioles 1-4	3	48	0.7266	0.7620	0.6593	Petioles 5 and above	1.1681	0.8646	0.6634
	4	49	0.8128	0.7956	0.6884		1.2731	0.9854	0.7285
	5	56	0.8625	0.8556	0.7899		2.2112	1.6226	1.2437
	6	63	0.9278	0.9047	0.8550		2.8516	2.4321	2.1056
			C v. WS: $P < 0.05$ at harvest 5.						

* Comparisons of C v. WS only are considered. These are significant at the level $P < 0.01$ unless otherwise stated. n.s.: not significant.

significantly lighter than control but petiole 2 is significantly heavier. The upper leaf group is significantly below control in both laminae and petioles. These trends are similar to those observed for WM in experiment 1 for the corresponding leaves and leaf groups.

At harvest 3 for WS the dry weights of the laminae and petioles of all leaf groupings are significantly less than control, but the difference is of greater magnitude in the laminae, and the level of significance achieved is higher for the upper petioles than for the lower. This response is similar to that in experiment 1, although the differences here are a little more pronounced.

At the final harvest for WM both the lamina and petiole of leaf 2 have regained control level but in leaf 6 they have not. The laminae and petioles of the four lower leaves are slightly below control but differences are more marked for the upper leaves. In the severely wilted plants, neither laminae nor petioles regain control levels upon re-watering.

(ii) *Relative Growth Rates*

For the laminae as a whole, the *R* values do not differ significantly at harvest interval 4-5 but at the final harvest interval 5-6, the comparison WS *v.* C is significant at the level $P < 0.001$, the values being: control = 3.83, WM = 4.67, and WS = 5.64 g/100 g/day. The significance attained in the total laminae at the final interval derives principally from the response of the upper laminae, the *R* values being 4.42, 5.90, and 7.27 g/100 g/day, and differing for the comparison C *v.* WS with high significance. Those for the group laminae 1-4, on the other hand, do not differ significantly (2.45, 2.15, and 2.23 g/100 g/day respectively). In this sense they agree with the trends of experiment 1. During wilting, *R* values for laminae 2 and 6 are depressed with high significance from 4.31 to 2.33 g/100 g/day in the one case, and from 9.38 to 5.37 g/100 g/day in the other case, for the comparison C *v.* WS. Upon recovery the trend is reversed in a manner similar to that of experiment 1.

(iii) *Water Content*

Figures 5 and 6 show the absolute and relative water contents of the laminae and petioles of leaves 2 and 6. Relative water contents fall markedly during severe wilting in these fractions, and moderate wilting causes a considerable drop in the absolute water content of leaf 6 fractions. Upon re-watering, relative water contents rise above control in the laminae and petioles of both leaves. Absolute water content upon re-watering attains control level in both the lamina and petiole of leaf 2, but does not in leaf 6. The data for the leaf groups are not presented because the individual leaf data are sufficiently representative of these fractions.

The values for relative water are similar to those of the corresponding fractions in experiment 1. The values for absolute water are different for lamina 6 at the final harvest, for in experiment 1 all the eight laminae attain control level at this stage. But the absolute water content of the fraction lamina 5 and above does not attain control level in either experiment, although the difference is greater in experiment 2.

IV. DISCUSSION

It is evident that in both experiments, leaves of differing position and age respond to treatment quite differently both during and after wilting. This is apparent from a consideration of the dry weights, the relative growth rates, and the water contents. The dry weight data for the four lower (older) leaves has been seen to differ in response to treatment from that of the four upper (younger) leaves, especially in experiment 1. These treatment responses are modifications of a pattern of translocation between plant parts similar to that observed by Goodall for the tomato at the eight-leaf stage.

Comparison of WM with control in both experiments shows that, at the conclusion of the water shortage, the upper laminae had been impaired in development much more than the lower. This suggests that translocation to the upper laminae had been impaired by wilting. However, the lower petioles were significantly greater in dry weight in WM than in the controls at this stage, but the upper petioles were not. It seems likely that dry matter was being translocated through the lower petioles at some stage of the wilting and might have been passing from the lower laminae to the stem. It has already been concluded (Gates 1955) that translocation of dry matter to the stem from the laminae was occurring during the course of wilting. It may therefore be concluded that in WM, translocation from the lower to the upper leaves was impaired during the course of wilting, but continued to the stem and may even have been enhanced by increased hydrolytic activity in the lower leaves.

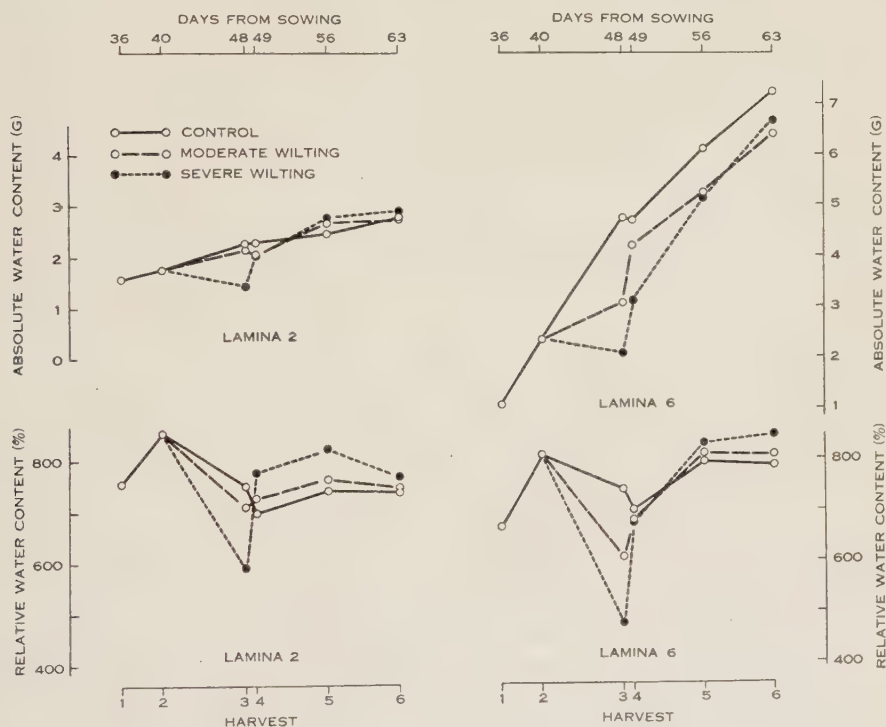


Fig. 5.—Water content of the laminae of the second and sixth leaves, experiment 2.

In WS, trends relative to control were of the same nature as in WM, but dry weight increment was less and may have masked the tendency to increased dry weight in the petioles. Translocation to the upper laminae was considerably impaired, but translocation to the stem from the laminae had been continued, and was principally from the lower laminae.

It is clear that the decreased lamina weight ratio and the increased stem weight ratio already noted (Gates 1955) for the whole plant during wilting,

arose because of modifications to the pattern of translocation between plant parts during wilting.

Although significances for the one day interval between harvests at re-watering cannot be established, the data suggest that translocation of dry matter from the lower laminae to the upper was resumed almost immediately upon re-watering.

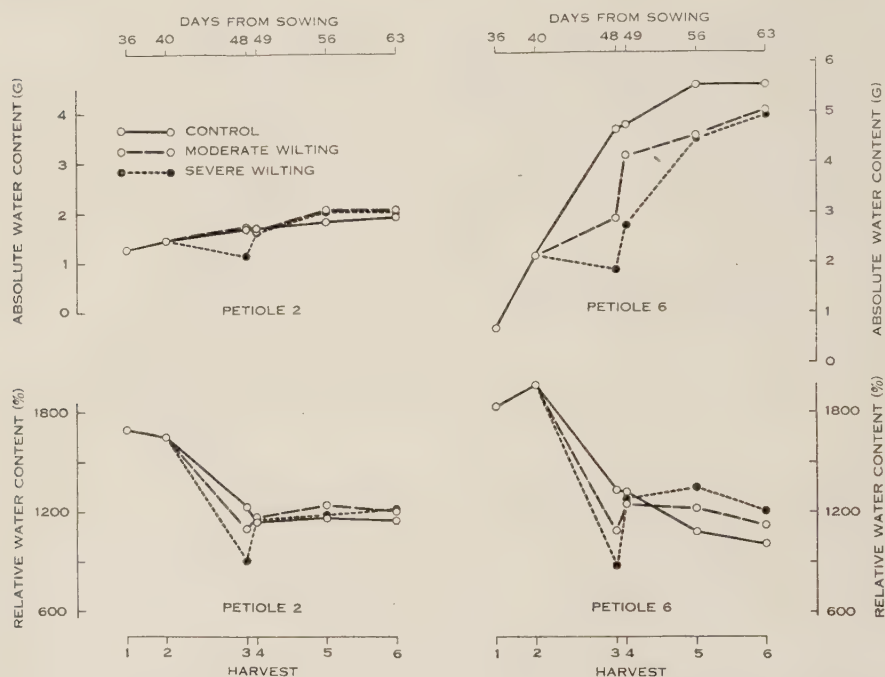


Fig. 6.—Water content of the petioles of the second and sixth leaves, experiment 2.

The relative growth rates in the two experiments are in agreement in showing that the reduction in R values for each lamina during wilting was to a similar proportion of the control value but was greater in absolute amount in the upper laminae. R values after wilting rose above control in a somewhat similar pattern, and the degree to which they rose above control increased with the severity of wilting. The greater changes in the upper laminae indicate that, both during and after wilting, development was most affected in the laminae that were growing most actively when the water shortage occurred. The fact that the more mature laminae showed a similar proportional change, may either mean that in their case the whole of a lamina developed slowly or that certain actively growing portions were the centres of response. Growth in more mature tomato laminae is most active in localized regions and especially where small leaflets are expanding. It is therefore most likely that it is the more actively growing portions of the lower laminae that are affected by water shortage, the effect occurring both during and after wilting, and in opposing senses. The fact that the treatment response was greater in the younger and most actively developing laminae, and possibly in the more actively developing

parts of the older laminae, suggests that the response might well be due to a reduction in the rate of cell expansion and development during water shortage, followed by the resumption of normal rates upon re-watering.

Petrie and Arthur (1943) studied the effect of early and late temporary drought and of permanent drought on the development of groups of leaves in tobacco. In their late temporary drought, soil moisture was reduced to below the permanent wilting percentage at the time of "maximum dry weight" of the leaves. At this stage, treatment hastened senescence, especially in the lower leaves, but there is a suggestion that the dry weights of the upper leaf group increased again after re-watering.

The resurgence of active growth in the laminae upon re-watering from the wilted condition is accompanied by a higher relative water content than that of control. The data for the individual leaves show that the relative water contents of the laminae and petioles after re-watering rise above control to such an extent that absolute values at the final harvest attain control level in all the first eight laminae and in almost all the petioles. However, the greater differences in relative water are in the younger laminae and petioles. During wilting, the greater differences in relative water were also in the younger laminae and petioles, although in the opposite direction. Thus, with respect to water content, the younger leaves are more affected by water shortage than are the older leaves, but the response of the younger leaves to renewed water supply is the more intense. The older leaves, however, behave in a similar manner if not to the same degree as the upper and, as was noted for the relative growth rates of the laminae, it seems likely that it is their younger portions that contribute most to that response.

It is clear upon considering the growth and water content data of the individual leaves that it is the younger portions of the total leaves that contributed very largely to the decline in active development that occurred for the whole plant during the water shortage. Upon recovery from the water shortage they were important centres for the response characteristic of the physiologically younger condition already observed for the whole plant.

The question naturally arises whether the set-back due to water shortage arises by reason of a failure to differentiate new tissues or a failure for already differentiated tissues to develop by cell expansion or by other means. The recovery phase might occur by either accelerated differentiation or accelerated development, or both. Morton and Watson (1948) found with sugar beet that the rate of formation of leaf initials was relatively insensitive to water supply, and that transition from high water treatment to low water hastened the appearance of senescence in leaves, the reverse change delaying it. Ashby (1948) studied the morphogenesis of leaves of *Ipomoea* and compared groups of plants grown under dry conditions with groups of plants grown under high humidity and adequate watering. He found that water supply to the leaf affected leaf area and cell number in the epidermis but did not affect cell size. He also found significant increases in leaf area, and in cell number but not in cell size, in the epidermis of leaves that developed soon after watering desiccated plants.

In experiment 1 no significant difference could be detected between leaf numbers at either harvest 4 or 6 in the comparison of control and WS. Similarly,

differences in the numbers of fruit trusses formed and the numbers of buds, flowers, and fruits (collectively) were not significant at harvest 6 between control and WS. But comparisons between control and WS of the numbers of visibly set fruit or of the numbers of opened flowers (whether with fruit or not) are in each case highly significant at harvest 6 and differ by approximately 100 per cent. It is reasonable to suppose that some stages of the differentiation of the inflorescences proceeded during the course of the wilting. If this is so, even though the evidence is far from conclusive, it would seem to suggest that differentiation of these tissues was not greatly impaired by wilting, but that the overall development of them was delayed.

V. ACKNOWLEDGMENTS

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PHYSIOLOGICAL GENETICS OF *PISUM*

I. GRAFTING EXPERIMENTS BETWEEN EARLY AND LATE VARIETIES

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Summary

The grafting of genetically early scions of *Pisum sativum* var. Massey on genetically late stocks (var. Telephone) leads to flowering at a higher node. In reciprocal grafts, the scions of the late variety flower at an earlier (lower) node.

Control grafting, i.e. scions to stocks of the same variety, has no effect on Massey scions but leads to flowering at a lower node in the Telephone scions.

Removal of cotyledons as soon as possible after germination (3-7 days) has no effect on Massey but leads to earlier flowering in Telephone.

These results are best explained on the assumption that the genetically later variety produces a flower inhibitor (or delaying) substance which can pass a graft union and alter the flowering behaviour of genetically early scions.

I. INTRODUCTION

The garden pea, *Pisum sativum*, is a classical subject for work in both genetics and physiology. In general, the work in any one of these disciplines has been carried out with little reference to the other. The exceptions to this general statement are few and concern studies on the physiological activity of genes controlling growth in length of the internodes. De Haan and Gorter (1936) attempted to show that the "slender" pea, which is the double recessive with respect to the two multiple loci *La* and *LB* (or, to use Lamm's (1947) symbolism, *Cy*₁ and *Cy*₂) differ from the normals in having a lower capacity for the enzymic destruction of auxin.

By means of reciprocal grafting of etiolated plants, Went (1938, 1943) has obtained some evidence that "slender" peas also differ from their normal tall (*Le*) or short (*le*) sisters in the production of, and response to, the caline group of hormones. He has shown that similar differences may exist between different commercial varieties. These last differences have not been investigated genetically. Went (1943) was unsuccessful in obtaining transmission of "acacia-leaf" (*tl*), "stipuleless" (*st*), and "rogue" (plasmagene) characteristics across graft unions between normal and mutant stocks.

Von Abrams (1953) who worked with two commercial varieties, Tall Telephone and Dwarf Telephone, which possibly differ at the *Le* locus, was unable to demonstrate any differences in the enzymic mechanisms controlling auxin production and destruction, although the etiolated dwarf responded more to auxin sprays than the etiolated tall.

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Commercial varieties of peas differ greatly in flowering behaviour. The genetics of these differences is complex but in view of our increasing knowledge of the physiological mechanisms controlling flowering (Lang 1952) a joint physiological and genetical study seems worth attempting. This paper gives an account of the results of grafting experiments between genetically early and late varieties. A preliminary account of the experiments has been published (Barber and Paton 1952). The results are most easily interpreted on the basis of a flower inhibitor (or delaying) substance which is produced by the late varieties and can pass a graft union and delay the flowering of a genetically early scion. The analysis of the genetical differences between the two varieties will be published separately.

II. MATERIAL AND METHODS

(a) Survey of the Flowering Behaviour of Some Commercial Varieties

Table 1 gives a survey of certain characteristics of the commercial varieties available in Hobart. The data refer to spring sowings in the experimental garden of the University. The characteristics that have been measured are (i) node number (N), exclusive of the cotyledonary node, at which the first flower forms, (ii) number of days (T) from soaking of seed to opening of first flower, and (iii) height of plant (H) to first flower.

It will be seen that the node number for these varieties varies from 9 for Massey to 18 for Telephone. This difference in node number corresponds to a difference of about 22 days in flowering time. While there is a significant linear regression of days to flower on node number ($T = 58.7 + 2.0 (N - 14.5)$, $P < 0.01$) this general comparison shows that some varieties, e.g. Alaska, Canner's Perfection (early), and Latefeast are well off the regression line, Alaska and Latefeast being later than expected on the basis of node number and Canner's Perfection being earlier. This means presumably that time to flower is not determined absolutely by node number. Earlier genetical work which has been reviewed by Wellensiek (1925) and Rasmusson (1935) has given rise to the same general conclusion. Wellensiek assumed at least two types of genetic control, one pair of genes altering the node number and another pair at a different locus controlling flowering time or the rate of development of these nodes. Rasmusson, who unfortunately did not count node number in his progenies, has suggested that half the genic variation of flowering time in his crosses can be explained by two major genes, one linked to the A locus (flower colour) and the other, either identical with the Le gene (dominant for greater internode length) or very tightly linked to it, the le allele being dominant for later flowering. The other half of the genic variation is probably due to modifiers. On the other hand, Pellew (1940) has suggested that a series of three multiple alleles, L , l_1 , and l_2 determine the three main classes of flowering behaviour, the dominant L gene determining later flowering (18th node).

The varieties we have used also differ in internode length, the extremes again being Massey with a mean internode length of 0.7 in. and Telephone with a mean length of 2.0 in. There is apparently no correlation of internode

length with earliness either measured in terms of node number of first flower or in days to opening of first flower.

The data in Table 1 were obtained by sowing all varieties at the same time. The effect of variations in such environmental factors as photoperiod and temperature, which are known to affect flowering behaviour in many species of plants, have also been investigated in the two extreme varieties, Massey and Telephone. In general, Massey is relatively unaffected in node number by photoperiod and temperature, e.g. vernalization at 4°C, while Telephone behaves as a quantitative long-day plant, the node number of first flower falling from about 20 under short days (*c.* 10 hr) to 15 under long days (*c.* 18 hr). Telephone also shows a small vernalization reaction, germination at 4°C for three weeks lowering the position of the first flower by one or two nodes. These reactions will be described in full in a later paper.

TABLE 1
FLOWERING CHARACTERISTICS OF PEA VARIETIES: SPRING SOWINGS 1951

Variety	No. of Plants <i>n</i>	Node No. of First Flower <i>N</i>	No. of Days from Sowing to Flowering <i>T</i>	Ht. of Plant to 1st Flower (in.) <i>H</i>
Massey	45	9.5±0.14	44.8±0.65	6.8±0.31
Alaska	49	10.0±0.08	57.5±1.92	19.4±0.54
Utility	56	11.1±0.08	46.8±0.41	9.2±0.30
King Edward	51	14.6±0.15	62.5±1.93	19.9±0.62
Canner's Perfection (early)	49	14.6±0.12	47.8±1.87	16.3±0.46
Latefeast	33	14.6±0.12	66.9±0.67	19.6±0.53
Stratagem	27	14.7±0.17	62.4±0.90	12.9±0.46
Greenfeast	34	16.1±0.20	62.2±0.65	12.2±0.44
Canner's Perfection (late)	55	16.6±0.14	60.9±0.48	21.4±0.51
Yorkshire Hero	27	16.8±0.25	62.9±0.86	18.2±0.68
Te Oroha	32	17.2±0.16	62.8±0.91	23.3±0.97
Telephone	34	18.2±0.18	66.4±0.91	35.9±1.29

(b) Experimental Techniques

For the grafting experiments, the two extreme varieties William Massey (early, dwarf) and Telephone (late, tall) were used in most experiments. For some of the first experiments, Richard Seddon, an intermediate variety flowering at the 13th or 14th node, was also used.

The dry seeds were surface sterilized in a weak solution of bleaching powder, washed, and soaked in aerated tap water for 8 hr at 23-25°C. Soaking ensures uniform germination and tests conducted during the course of the present experiments have shown that over these short periods, the leaching action which Bonner, Haagen-Smit, and Went (1939) and Eyster (1940) have studied, does not affect germination, growth, or development of the peas.

Germination conditions were standardized, the turgid disease-free seeds being sown in fresh moistened sphagnum moss in seed boxes kept in a heated glasshouse at a temperature of 15-20°C. At the end of 3-4 days, when the plumules have grown to approximately 1 cm, healthy uniform seedlings were planted in 6 in. pots, six plants being allowed to mature. We have found it advantageous to leave the cotyledons exposed on the surface to facilitate easy access to the cotyledonary buds during post-graft inspections and also to reduce susceptibility to attack by pathological organisms. The plants were grafted 1-2 days later, when the epicotyl was approximately 2 cm and the second internode is just visible.

A cleft graft technique was used, the stock plant being decapitated immediately below the first leaf, care being taken to remove all bud tissue, which, if left on the stock, may proliferate from the cut surface. A median longitudinal cut for approximately half the length of the stock epicotyl allowed the wedge-shaped scion to be easily pushed into position. For strapping the cut surfaces together, thin rubber rings cut from bicycle valve rubber tubing is all that is required, the rubber ring being slipped over the stock just before insertion of the scion. With this method, it has been possible to obtain over 90 per cent. successful grafts and to complete an individual graft in less than a minute.

The grafted plants were protected from excessive transpiration for one to two days, by which time partial tissue union had taken place and water transport to the scions established. Even though tissue union was relatively rapid, there was no marked scion growth for 10-14 days. Indeed, establishment of full physiological union, including the organization and transport of factors responsible for apical dominance over lateral shoot growth, required a period of the order of 3-4 weeks (cf. Went 1943). Until apical dominance had been established by the scion apex, scion growth was slow, and the cotyledonary buds of the stock produced vigorous basal shoots. These shoots were removed daily until the scion had established dominance.

Thus grafting stops growth for a variable period. We have no evidence that the variation in time for a graft to unite directly alters node number. It does, of course, alter flowering time which is more variable in the grafted plants. For this reason, the results of the grafting experiments are analysed solely in terms of node number, exclusive of cotyledonary node, of first flower. Even in ungrafted plants the node number is a less variable quantity than days to flower, the coefficients of variation being 6 per cent. for node number and 11 per cent. for days to flower.

In addition, the effect of removing cotyledons has been investigated. Under the conditions of our experiment, it was found that the removal of cotyledons was fatal if performed earlier than three days after soaking. After this time the embryo can survive and flower in standard potting soil, the survival rate being proportional to the time after germination at which cotyledons were removed.

Spring sowings were used for the experiments. In view of the variation in flowering behaviour caused by environmental factors, a series of controls both grafted and ungrafted was used for each experiment.

III. RESULTS

The results of three grafting experiments are given in Table 2, and graphically in Figure 1. The more extensive data refer to grafting of the varieties Massey and Telephone. In the two experiments with these varieties we can compare the behaviour of ungrafted plants with their behaviour (i) when grafted on stocks of their own variety (control graft), (ii) when grafted on stocks of the other variety (experimental graft), and (iii) when the cotyledons are removed as early as possible and without grafting.

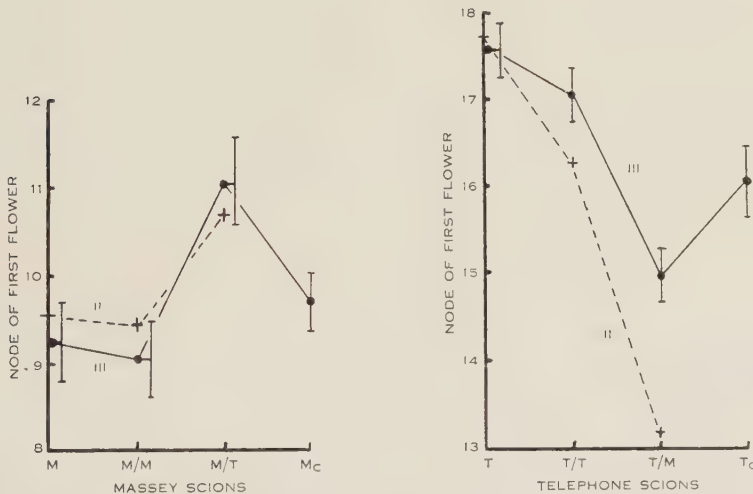


Fig. 1.—Diagrams showing the influence of control grafting (M/M and T/T), experimental grafting (M/T and T/M), and removal of cotyledons (M_c and T_c) on node of first flower of scions of Massey (M) and Telephone (T). The results of experiments II and III are given. The lengths of the vertical lines for experiment III represent twice the standard errors of the mean. For the sake of clarity they are not shown for experiment II but may be seen in Table 2.

In the case of Telephone, all three treatments result in significantly earlier flowering in both experiments. The greatest decrease in flower node is obtained by grafting to Massey stocks, the mean node number being significantly reduced by 4.6 nodes and 2.6 nodes. Control grafting gives the least reduction in node of first flower (1.4 and 0.5 nodes), the smaller of the two differences being statistically significant at the 1 in 50 level of probability.

As regards Massey scions, both experiments show that grafting on Telephone stocks leads to significantly later flowering, whilst control grafting has no significant effect. In the first experiment, removal of cotyledons has apparently caused later flowering, the difference being statistically significant at the 1 in 100 level of probability. However, this result is based on five plants only. In the second experiment, based on 38 plants, there is no significant difference in flowering node between intact plants and plants with cotyledons removed.

Table 2 gives the results of grafting the two varieties Richard Seddon and Telephone. Richard Seddon is an intermediate variety flowering at the four-

TABLE 2
RESULTS OF THREE GRAFTING EXPERIMENTS

The table shows the number of plants (n), the node of first flower (N), and the standard error of the estimate of the mean (SN)

GRAFTING EXPERIMENT I. SEPT.-DEC. 1949

Experimental Treatment of Scion	Richard Seddon Grafted on Telephone		Telephone Grafted on Richard Seddon	
	n	$N \pm SN$	n	$N \pm SN$
Control, ungrafted	40	13.73 ± 0.101	40	16.78 ± 0.141
Control, grafted	19	13.95 ± 0.209	17	15.94 ± 0.218
Experimental graft	13	$14.46 \pm 0.186^{***}$	23	$14.83 \pm 0.150^{***}$

GRAFTING EXPERIMENT II. SEPT.- DEC. 1950

Experimental Treatment of Scion	Massey Grafted on Telephone		Telephone Grafted on Massey	
	n	$N \pm SN$	n	$N \pm SN$
Control, ungrafted	36	9.53 ± 0.093	23	17.74 ± 0.201
Control, grafted	44	9.45 ± 0.106	34	$16.29 \pm 0.201^{***}$
Experimental graft	18	$10.72 \pm 0.30^{***}$	17	$13.18 \pm 0.196^{***}$
Cotyledons removed, ungrafted	5	$10.80 \pm 0.583^{***}$	2	$15.50 \pm 0.500^{**}$

GRAFTING EXPERIMENT III. NOV.-FEB. 1950-1951

Experimental Treatment of Scion	Massey Grafted on Telephone		Telephone Grafted on Massey	
	n	$N \pm SN$	n	$N \pm SN$
Control, ungrafted	45	9.27 ± 0.215	63	17.59 ± 0.141
Control, grafted	24	9.04 ± 0.212	36	$17.08 \pm 0.141^*$
Experimental graft	31	$11.10 \pm 0.251^{***}$	63	$14.98 \pm 0.148^{***}$
Cotyledons removed, ungrafted	38	9.71 ± 0.164	51	$16.06 \pm 0.195^{***}$

The significance of difference between means of ungrafted controls and treatment means is indicated at the 0.05 level of probability (*), 0.01 level of probability (**), and 0.001 level of probability (***).

teenth node. All treatments lead to significantly earlier flowering in Telephone, but, when Richard Seddon is grafted on Telephone, it flowers significantly

later. The differences in all cases are less than those in the comparable Massey-Telephone experiments.

A few observations have been made on the effect of varying the time interval between germination and grafting. In Experiment II, the time of grafting varied between 7 and 12 days after soaking the seed. There was a gradual loss in the response of Massey scions to grafting on Telephone stocks over this period, 12-day-old scions flowering at the same node as the ungrafted controls or control graft. This loss of flexibility is not shown by Telephone scions, these showing similar decreases in node number whether grafted on Massey at 7 or 12 days after soaking.

Both varieties have 5-7 nodes laid down in the dry seed. Flower primordia are detectable in Massey at the ninth or tenth node 10-15 days after soaking, whereas Telephone takes at least 30 days to form flower primordia. Thus the delaying effect of a Telephone stock on a Massey scion becomes ineffective once the flower primordia are laid down.

IV. DISCUSSION

The results are best interpreted on the assumption that flowering behaviour in these pea varieties is mainly determined by the production of a flowering inhibitor in the cotyledons of late varieties which is then transported to the plumule. There are three lines of evidence in favour of this hypothesis. (i) Grafting of early scions on late stocks delays flowering in the early scion, while in the reciprocal graft, flowering in a late scion is hastened by an early stock. This result could equally well be interpreted in terms of a flowering stimulus produced in Massey. However, the other two lines of evidence show that this action is due largely to a flowering inhibitor in the late varieties. They are: (ii) removal of cotyledons from Massey has no effect on flowering, whereas the removal of cotyledons from Telephone causes flowering at a lower node; and (iii) control-grafting, which must interrupt the supply of inhibitor to some extent, results in earlier flowering of Telephone but has no effect on Massey.

There is some slight evidence that the cotyledons of Massey may also contain a substance promoting flowering. The best evidence is, perhaps, that removal of cotyledons in Telephone is apparently less effective in lowering the node of first flower than is grafting on Massey stocks. However, this evidence is of doubtful significance since in order to obtain sufficient scorable plants after removal of cotyledons, the plumules had to be left longer in contact with their own cotyledons than if the plumules were grafted onto Massey. Haupt (1952) has obtained a slight indication that removal of cotyledons within 8 hr of soaking the seed of the early variety, "Kleine Rheinlanderin" (node number 9-10), may lead to a delay in flowering. His experiments were again based on a small number of plants and were apparently significant only between the 0.05 and 0.01 levels. As indicated above in one of our experiments a similar effect was noted but it has not yet been repeated.

As regards the properties of the inhibitor, it is clear that a vegetative plumule can remain reactive to it only over a relatively short time, or that the cotyledons soon stop producing inhibitor. The inhibitor cannot alter flowering behaviour in Massey once flower primordia have appeared, i.e. 10-12 days after soaking. Telephone scions apparently remain reactive for more than 12 days. As regards the time during which the cotyledons produce inhibitor, preliminary experiments indicate that production stops after about 14 days, i.e. when most of the food reserves have been removed from the cotyledons.

This short period over which inhibitor production is active provides the explanation for the earlier flowering of control grafts in Telephone. While water transport is established within 3 days of grafting, full growth rate and apical dominance in the scion are not established for a period of 2-3 weeks. Usually apical dominance, as shown by cessation of growth of cotyledonary buds, is established before maximum growth rate is reached. It is unlikely that a 3-day interruption in supply of inhibitor would result in an earlier flowering of 1-2 nodes, so it appears probable that the transport of the inhibitor requires the slower and more intimate physiological union associated with the development of apical dominance. No direct attempts have been made to see whether the inhibitor can pass a water barrier in sufficient quantities to alter the flowering behaviour of early scions.

Comparison of the effect of Richard Seddon and Massey stocks on Telephone scions shows that Richard Seddon is about half as effective in lowering the node of first flower. This presumably means that Richard Seddon stocks produce some inhibitor but only about half as much as Telephone stocks.

The inhibitor is probably of a hormonal nature. Haupt (1952), using "Kleine Rheinlanderin," has shown that extracts of yeast, pea cotyledons, etc., contain substances which can delay the flowering of embryos from which the cotyledons were removed after a few hours of soaking in water. The embryos without cotyledons in these experiments were then grown to the stage of flower initiation on a synthetic medium of agar, sucrose, and mineral salts to which the various extracts were added. Cruickshank (unpublished data) has repeated some of Haupt's experiments using Massey and isolating the embryo aseptically from the cotyledons in the dry seed. He usually finds no delay in flowering if the cotyledons are removed in this way and the embryos grown in artificial light on the synthetic agar medium. He confirms that yeast extracts and extracts from Massey and Telephone seeds contain inhibitors increasing the node number from about 9.5 to 12. However, no differential effect of extracts from the two varieties could be demonstrated and attempts to fractionate the extracts into ether and water soluble fractions have failed. These experiments are continuing with the use of diffusion methods of extraction similar to those used by Bonner *et al.* (1939) in their search for leaf growth factors.

As regards chemical treatments, which might give some indication of the nature of the inhibitor, there appears to be increasing evidence that auxins (indoleacetic acid (IAA), or naphthaleneacetic acid (NAA)), will inhibit flowering in short-day and photoperiodically indeterminate plants (von Denffer 1950; Bonner and Liverman 1953). In long-day plants responses are more

variable, although here an inhibition appears to be usual when treatments with auxins are given under long days. However, under short-day conditions just below the flowering threshold, auxin application may promote flowering (Bonner and Liverman 1953). Earlier work, e.g. Leopold and Thimann (1949), has indicated that an optimum auxin concentration may exist in certain species. Borgstrom's (1939) results with an early variety of pea grown in darkness, appear to indicate that a concentration of IAA of 1 p.p.m. may be the optimum concentration as regards earliness and number of flowers under these growth conditions. Leopold and Guernsey (1953, 1954) using the early variety Alaska have shown that the effect of auxin treatment depends in a complex way on the temperature and light intensity at which the plants are grown after treatment. The auxin (NAA) was applied by soaking the seed in solutions for 4-18 hr. If the seeds are then grown at 20°C, the auxin over a concentration range of 10^{-7} - 10^{-3} M, delays flowering by 2-3 nodes. If the auxin treatment at 10^{-5} M is followed by growth for a few days at 10°C, the flowering node is lowered. This effect is evident only at low light intensities (400 f.c. and below). Higher auxin concentrations are inhibitory no matter what the temperature and light regimes are.

Slade and Paton (unpublished data) have obtained no consistent results following auxin (IAA) and anti-auxin (2,3,5-triiodobenzoic acid) applications to seed and growing plants (lanolin paste or spray) of Massey and Telephone. The plants in these experiments were grown under ordinary glasshouse conditions. These observations make it doubtful whether auxin is responsible for the graft transmissible inhibition. Moreover, Haupt (1952) has shown that the floral inhibition in embryos without cotyledons by yeast extracts, is not replaced, nor is the inhibitory action of the extracts altered by addition of IAA at concentrations of 0.25 p.p.m. Thus, although we cannot at present exclude the possibility that an auxin activity is responsible for most of the graft transmissible flower inhibition, it appears more likely that the inhibitor formed by genetically late peas is not the natural auxin, IAA.

V. ACKNOWLEDGMENT

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CENTROMERIC BEHAVIOUR OF THE UNIVALENTS IN TWO *PHALARIS* HYBRIDS

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Summary

The hybrid between *Phalaris coerulescens* ($2n=14$) and *P. minor* ($2n=28$) usually forms seven bivalents and seven univalents at the first metaphase of meiosis. The univalents are derived from *P. minor*. The centromeres of the seven univalents do not divide at the first anaphase, but neocentromeres in distal parts of the chromosomes assume control of their movement. These neocentromeres move towards opposite poles, between which the univalents become stretched. Formation of the cell wall causes breakage of the univalent bridges. Fusions may occur between fragments or between broken ends of sister chromatids in a fragment. Neocentric activity occurs only infrequently at the second anaphase.

This pattern of univalent behaviour is characteristic of this particular genome.

The hybrid between *P. tuberosa* ($2n=28$) and *P. minor* ($2n=28$) has several univalents at the first metaphase. Some of these may show misdivision of the centromere at both divisions of meiosis, and occasional neocentric activity distally in the chromosomes. Usually the centromeres of the univalents divide normally.

I. INTRODUCTION

The movement of chromosomes on to, and of daughters away from the equatorial plane during cell division, is normally a reaction between the spindle and the centromere. The centromere has a special cycle of division at meiosis, remaining undivided until anaphase of the second division. At the first division of meiosis the two centromeres in the chromosomes of a bivalent co-orient.

Centromere behaviour at meiosis is modified for chromosomes which are univalents. They cannot co-orient, but may congress on the equator after the bivalents have disjoined. The centromeres of univalent chromosomes which have congressed usually divide at the first division. The daughter chromosomes do not congress at the second division and may enter a T II group or be left out and form a micronucleus. If a univalent fails to congress, it may be included in one polar group at T I when the centromere divides normally at the second division, or be left to form a micronucleus.

While this represents the usual pattern of behaviour, the centromeres of univalents may misdivide, as has been described previously (Upcott 1937; Sears 1952). Misdivision occurs in one of the *Phalaris* hybrids discussed in the present paper.

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In a number of instances, regions of the chromosome other than the centromere have assumed control of anaphase movement on the spindle after congression. Such regions may be conveniently referred to as neocentromeres (Rhoades 1952). In *Zea mays* (Rhoades and Vilkomerson 1942; Rhoades 1952), in *Secale cereale* (Kattermann 1939; Müntzing and Prakken 1942), and in *Elymus weigandii* (Vilkomerson 1950) this behaviour concerns bivalent chromosomes. Only univalent chromosomes are affected in an asynaptic *Secale* (Prakken 1943), in *Bromus* hybrids (Walters 1952*a*, 1952*b*, and unpublished data), and probably in an asynaptic *Pisum* (Koller 1938), in a haploid *Godetia* (Håkansson 1940), and in a *Pennisetum* hybrid (Krishnaswamy and Raman 1953).

With the possible exception of the *Pennisetum* hybrid, the true centromeres divide after the neocentromeres have moved some distance towards the spindle poles.

Both the hybrids to be described in the present paper exhibit neocentric behaviour. In one of them the centromeres do not divide, so leading to bridges at the first anaphase of meiosis.

II. MATERIALS AND METHODS

Dr. E. M. Hutton's *Phalaris* hybrids (Hutton 1953) were made available for examination.

Whole panicles were fixed in Bradley's (1948) fixative, which consists of four parts of chloroform, three parts of ethanol, and one part of glacial acetic acid, and stored in a refrigerator until used. Meiosis was examined using iron-aceto-carmin squashes made permanent by the method described by Darlington and La Cour (1947).

The somatic chromosome number of *P. tuberosa* L. and *P. minor* Retz. is 28, that of *P. coerulescens* Desf. is 14. The chromosomes of all three species have median or submedian centromeres. Meiosis and mitosis are normal. *P. minor* has seven large and seven small bivalents at M I, *P. coerulescens* has seven small bivalents, and *P. tuberosa* has 14 bivalents which exhibit a range in size.

III. RESULTS

(a) Meiosis in *P. coerulescens* × *P. minor*

Pachytene chromosomes are long and crowded, and paired and unpaired chromosomes cannot be traced completely. At M I the chromosomes are most frequently associated as seven bivalents and seven univalents (Plate 1, Fig. 1). The seven univalent chromosomes are conspicuously longer than the other 14 and are readily identifiable from M I onwards. They are evidently derived from *P. minor*. The mean chiasmata frequency per cell of the seven bivalents calculated on 143 cells is 14.40 ± 0.08 . This indicates a considerable degree of homology.

The seven large univalents may take part in other configurations at M I. They form three types of rod bivalent, one of which has a chiasma between

relatively inverted segments. In addition, a linear trivalent is common. This consists of two small chromosomes with a large one in a terminal position.

At A I, all the bivalent chromosomes disjoin and pass to the poles. Their behaviour is normal and is not discussed further.

(b) *The Behaviour of the Univalents at the First Meiotic Division*

The univalents not included in polar nuclei at A I congress in the equatorial plane (Plate 1, Fig. 2). Their chromatids fall apart but remain attached at the undivided centromeres. Distal parts of the chromatid arms become directed towards the poles, the univalents coming to lie axially rather than transversely in the spindle (Plate 1, Fig. 3). The types of univalent orientation are shown in Figure 1. Two chromatid arms may be directed towards each pole (Figs. 1a and 1b) or one chromatid arm may be directed to one pole and three to the other (Fig. 1d). Univalents have been seen in which one or more of the chromatid arms appear to remain passively in the equator and contrast sharply

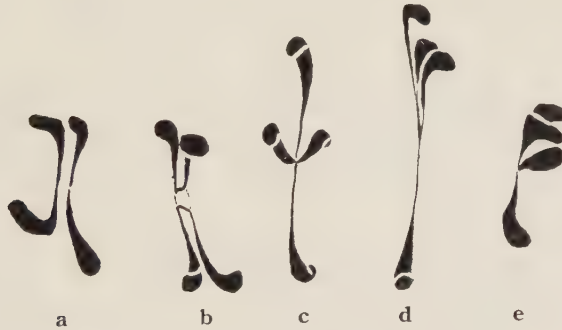


Fig. 1.—*P. coerulescens* × *P. minor*. Univalents at A I showing neocentric activity. × c. 1500.

with those that have moved poleward (Fig. 1e). Usually, however, all arms of all univalents that have congressed are affected, although the activity of the neocentric regions, presumed to be responsible for this orientation, varies so that one arm may be more stretched than the others (Figs. 1c and 1d).

The ends of the affected chromatid arms are always reflexed suggesting a subterminal location for the neocentromeres (Plate 1, Figs. 3 and 4). As the univalents are morphologically similar, no one chromosome is identifiable from cell to cell. Comparisons of the length of proximal stretched, and distal unstretched segments of an arm are difficult to make reasonably accurately. Thus it has not been possible to demonstrate a constant location for the neocentromeres in each particular chromosome. However, sister arms normally correspond in appearance.

In some univalents it was possible to trace the chromatids through the centromere and to see that when two chromatid arms pass to each pole they may be either sisters or non-sisters (Figs. 1a and 1b). The number of univalents

with three arms directed towards one pole and one toward the other pole, varies between cells. Where there are two such chromosomes in the same cell, the three arms may or may not be directed towards the same pole. It appears therefore, that the influence of the neocentromeres on the orientation of the univalents is irregular, and that sister neocentromeres do not always disjoin from one another. There are three arrangements of the arms of the congressed univalents: (i) all four arms directed to one pole, (ii) three arms directed to one pole and one to another, (iii) two arms directed to each pole. If the distribution of the arms under the influence of the neocentromeres is random, the frequencies of these three classes will be given by the sum of the appropriate terms in the expansion of the binomial expression $(\frac{1}{2} + \frac{1}{2})^4$, namely $\frac{1}{8}$, $\frac{1}{2}$, and $\frac{3}{8}$ respectively. Identification of class (i) is uncertain. When this class is ignored and the chromosomes in the other classes counted, 98 chromosomes are of class (ii), and 149 are of class (iii). The numbers expected in these classes, assuming random orientation are 141 and 106 respectively. There is a highly significant departure from expectation, χ^2 being 30.7 for 1 degree of freedom.

It appears that the chromatid arms tend to go in pairs. This is probably due to initial orientation on the metaphase plate and also to repulsion between chromatid arms.

Univalents may be included in a polar group by the greater activity of one of the sets of neocentromeres, perhaps assisted by initial displacement from the equatorial plane. No first division cells were seen where the neocentromere of one chromatid exerted sufficient activity to draw three opposing chromatids over the midpoint of the cell.

At the end of A I, from one to seven univalents lie stretched across the cell. The average number is between four and five. The median portion of the stretched chromatids near the equator of the spindle tends to become very slender. Some breakage by actual tension may occur at this stage, but most, if not all the breakage observed later, is due to the formation of the cell wall.

The precise sequence of events leading to cell wall formation is somewhat uncertain and may be variable. When there are a number of univalent bridges, both polar groups and the univalents are frequently enclosed in the one nuclear membrane. As the cell wall forms, the cytoplasm appears to withdraw on either side. The presence of a group of univalent bridges between the polar groups apparently prevents the withdrawal of the cytoplasm in the centre of the cell, while the cytoplasm all around the univalents is cleared (Plate 1, Fig. 5). Pressure from the cell wall formation appears to be exerted at the centre of the cell on the univalent bridges, compressing them so that there is a slender waist. Further wall development usually results in breakage of the univalents (Plate 1, Fig. 6).

The stretched univalents sometimes prevent the formation of the cell wall so that a restitution nucleus is formed. Cells with 21 chromosomes at each pole have been observed at A II. Their frequency makes it unlikely that they were derived from areas of tetraploid tissue, no evidence of which has been seen in any of the first division cells.

Even after cell wall formation is completed, chromosome connections between the cells may persist for some time. Where only one or two univalents lie across the cell, the formation of the cell wall proceeds unimpeded.

(c) *Univalent Behaviour at the Second Meiotic Division*

The effects of univalent breakage on second division configurations are summarized in Figure 2, which includes all the types which have actually been observed.

The most obvious products of univalent breakage are chromosomes with three arms (type V) or with three normal arms and a small fourth arm (type IV). Acentric fragments (types I, II, and IV) are very frequent. Dicentric chromosomes (type II) have been observed in only a few cells. They could also be due to the inversion pairing mentioned previously.

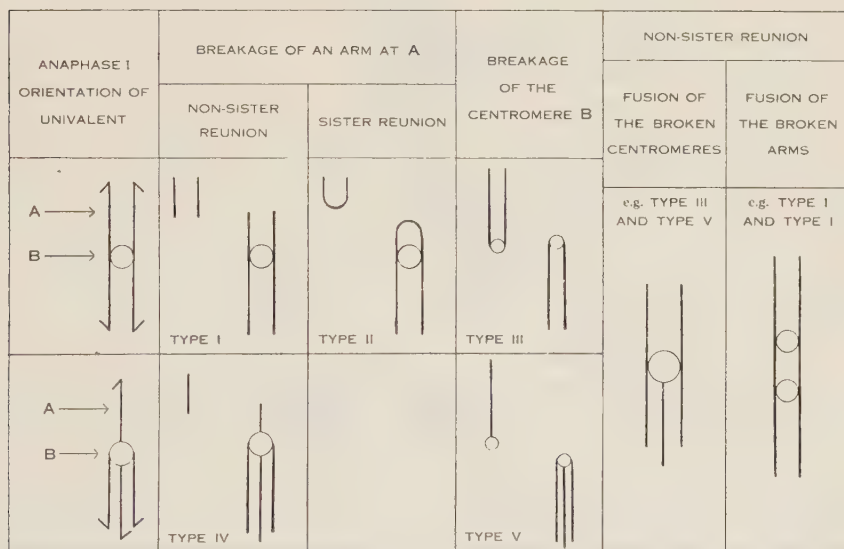


Fig. 2.—*P. coerulescens* \times *P. minor*. Diagrams of the products seen at second division after univalent breakage and reunion at the first division.

Occasionally a remarkable multi-armed chromosome was seen. The number of arms varied from five to eight and in some cases was perhaps more. The chromosome arms were of similar size and were usually grouped radially about a central non-staining region (Plate 1, Figs. 8 and 9). In some cells it was evident that the structure had two or three centromeres lying close together, but in other cells two or three centromeres could have been fused together. It is believed that these multi-armed chromosomes are the result of fusion between the broken ends of chromatids or broken centromeres, which arose when univalent bridges compressed together were broken by the cell wall at T I. The fate of these chromosome bodies is uncertain but they appear to form micronuclei.

Figure 3 shows a pollen mother cell at late A II, the seven chromosomes in outline at each pole being those derived from the seven bivalents. The other chromosomes are those derived from the seven univalents. Those labelled A, B, and C are chromatids from unbroken univalents which were included in a polar group at the first division, and which have divided normally at A II. The D chromosomes are the result of type III breakage. The E chromosomes are



Fig. 3.—*P. coerulescens* \times *P. minor*. Pollen mother cell at A II showing breakage and reunion products of the univalents. \times c. 1200.

the result of type IV or type V breakage, the breakpoint being adjacent to the centromere. The F and G chromosomes are the result of type I breakage. Instead of fusion between sister chromatids, fusion has taken place between adjacent centric fragments of non-sister chromosomes (cf. Fig. 2). The four acentric chromatid arms of F and G remain inert on the other side of the plane of first division.

Those univalents included unbroken into one of the polar groups at T I disjoin normally at A II. There is no appearance of neocentric activity in these chromosomes, nor in those which belong to type I. Some of the products (types III, IV, and V) of univalent breakage at the first division remain behind

at the second when the normal chromosomes have disjoined, and behave in various ways. Type III chromosomes are the only ones which display neocentric activity at the second division, and not all such chromosomes show it. The neocentromeres appear in both arms and the chromosome may lie axially in the cell with one arm extended to each polar group (Plate 1, Fig. 7). These chromosomes are usually included in one of the daughter nuclei presumably through the greater activity of a particular neocentromere, or rarely such chromosomes remain between the polar groups to be broken by the second division cell wall. Rarely both chromosome arms are directed towards one pole and the true centromere towards the other pole (Fig. 4). Thus the broken centromere



Fig. 4.—*P. coerulea* \times *P. minor*. A II showing joint activity of centromeres and neocentromeres in three univalents. Two acentric fragments are present. \times c. 3000.

of these chromosomes is not inert as it was in the first division. When type III chromosomes do not show neocentric behaviour, they frequently divide, or misdivide, at the centromere. The exact nature of this division depends on the non-homology or homology of the chromosome arms, and presumably both occur.

The centromeres of type V chromosomes do not divide at the second division, and the chromosome remains in the middle of the cell. Type IV chromosomes were not observed at A II.

Very few pollen grain divisions were observed as most of the pollen grains failed to survive long enough to divide. Consequently, the later behaviour of the broken chromosomes could not be traced.

(d) Meiosis in *P. tuberosa* \times *P. minor*

The association of the chromosomes is very variable at M I. Linear trivalents occur in 90 per cent. of the cells and there are between eight and nine univalents on the average per cell. The univalents vary considerably in size and no one chromosome could be identified as derived from a particular parental

species. The behaviour of the bivalent and multivalent chromosomes at meiosis is normal, and when they have disjoined at A I, varying numbers of univalent chromosomes congress at the equatorial plane. The various types of behaviour of the univalents with their corresponding frequencies are given in Table 1.

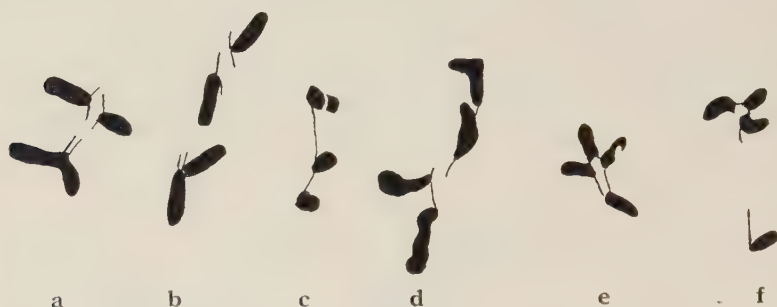


Fig. 5.—*P. tuberosa* \times *P. minor*. Univalents showing various types of misdivision of the centromere. $\times c. 1300$.

Misdivision of the centromere occurred in 20 of the 38 A I cells examined. Only one or two chromosomes in a cell misdivided, rarely more, and these included both large and small univalents. Three types of misdivision were observed (Fig. 5). The first involved separation of one chromatid arm from the centromere (Figs. 5e and 5f). The second type followed normal division of the centromere, the centromere of one of the daughter chromosomes divid-

TABLE 1
BEHAVIOUR OF UNIVALENT CHROMOSOMES AT MID-ANAPHASE I IN THE HYBRID *P. TUBEROSA* \times *P. MINOR*

Centromere Fails to Divide	Centromere Divides Normally		Centromere Misdivides		Total
Neocentric Activity	Neocentric Activity	No Neocentric Activity	Neocentric Activity	No Neocentric Activity	
No. of univalents: 23 Per cent.: 8.6	110 41.7	101 38.5	18 6.8	12 4.5	264

ing transversely to give two telocentric arms (Figs. 5a, 5b, and 5c). The third type of misdivision resulted in four telocentric arms (Fig. 5d) presumably by an extension of the second type, whereby the centromeres of both daughter chromosomes misdivide. Thin non-staining connections were visible between some of the misdivided centromeres. All these features have been observed by other workers. Misdivision of the centromere of single chromosomes was seen in 16 out of 50 A II cells.

Neocentric activity at the first division was observed in both large and small univalents and occurred in 90 per cent. of the cells examined. The range in appearance of the chromosomes with neocentromeres (Fig. 6) was greater than in the hybrid *P. coerulescens* \times *P. minor* (Plate 1). Usually the end of the chromatid arm directed towards the spindle pole was rounded. No thin attenuated ends of the chromosomes like those in *Bromus* hybrids (Walters 1952*b*) were observed. Frequently only some of the chromatid arms of a univalent were affected. It could not be decided with any certainty whether sister arms were directed towards the same pole or not. Usually the centromere of the univalents divided normally after the neocentromeres had acted. Univalents which had misdivided, frequently showed neocentric activity in the telocentric and metacentric arms. Thin faintly-staining connections similar to those seen in the misdividing univalents, were sometimes present between univalents whose centromeres had apparently divided normally, and which had shown neocentric activity.



Fig. 6.—*P. tuberosa* \times *P. minor*. Univalents at AI showing neocentric activity. \times c. 3000.

At the second division of meiosis neocentric activity of the chromatid arms was observed in 15 of the 50 AII cells examined. Various types of orientation result from this activity (Plate 1, Fig. 11), with distal or terminal regions of the chromosome arms leading towards the poles. No cell was seen where both arms of the same univalent were directed towards the same pole.

Thirty per cent. of the TI cells examined possessed micronuclei. These were entirely absent from the hybrid first described. The micronuclei appear to be derived from univalents and telocentrics which failed to reach the polar groups.

IV. DISCUSSION

Cell wall formation appears to be retarded or inhibited by several univalents stretched across the cell in *P. coerulescens* \times *P. minor*. A similar obstruction of cell wall formation has been described in *Triticum* hybrids (Thompson 1931). The complete prevention of cell wall formation is rare in the *Phalaris* hybrid, even when there are a large number of univalents.

The bridges which usually lie across cells are chromatid bridges, and are broken by the cell wall. Dr. O. H. Frankel has suggested to me that the essential difference in action on cell wall formation between chromatid bridges and univalent bridges resides in the position of the centromere. Possibly it is the univalent's centromeres lying in the middle of the cell which impede, and occasionally prevent, cell wall formation.

The multi-armed chromosomes observed at the second meiotic division in *P. coerulescens* \times *P. minor* are unique. They may be the result of reunion during the interphase, after chromosome breakage at cell wall formation at the first division. They are different from diplochromosomes (White 1935), which have eight chromatids with a single centromere.

It is evident that chromosomes at the second division which lie axially in the cell and show neocentric activity of the distal parts of the chromatid arms are not instances of misdivision of the centromere. Upcott (1937) and Sanchez-Monge (1950) have supposed similarly orientated chromosomes to be undergoing transverse division of the centromere.

The common feature of the neocentric activity shown by the univalents of the *Phalaris* hybrids and the previously reported examples, is the presence of lagging univalents at meiosis in which neocentric activity may occur.

There is no evidence of a visible "activator" of neocentric activity such as the abnormal chromosome 10 in *Zea mays*. The particular condition which evokes neocentromere activity may be either a genetic unbalance, or an alteration of the spindle by the centromeres of the disjoined chromosomes as suggested by Darlington (1937).

In *Zea* and less definitely in *Secale* it has been shown that there are constant locations in the chromosomes for the neocentromeres, and that their loci are heterochromatic. Although there is no evidence for the heterochromatic nature of the neocentromeres in the *Phalaris* hybrids, the constancy in appearance and behaviour of the univalents in *P. coerulescens* \times *P. minor* strongly suggests a constant location and a common structure of the neocentromeres. The difference between the degree of neocentric activity in these two hybrids may then be the result either of genotypic differences or of structural differences or a combination of both.

The acentric fragment derived from the inversion occasionally seen in *P. coerulescens* \times *P. minor* has not been observed at AI. Hence Rhoades's (1952) observation that the true centromere is essential for the functioning of the neocentromere in *Zea* has not been capable of test in this material.

While the large univalents in *P. tuberosa* \times *P. minor* cannot be assigned with any certainty to either parent, it is significant that univalent bridges occur in this hybrid, similar to those seen in the first hybrid. It is not unreasonable to suppose that these univalent bridges are derived from the large chromosomes of *P. minor* and that the centromeres of this particular genome have a particular pattern of response to the univalent condition. This supposition is favoured by the behaviour at AI of the occasional univalents in allopolyploid *P. coerulescens* \times *P. minor*. The large univalents derived from *P. minor* behave like those in the undoubled hybrid. The small univalents from *P. coerulescens*, how-

ever, do not show this extreme abnormality. Further, the univalents in an asynaptic *P. coerulescens* behave normally.

The loss of that property by which the centromeres lead chromosome movement is correlated with irregularities in the division of the centromere in the univalents of the *Phalaris* hybrids, in a *Bromus* hybrid (Walters 1952a), and apparently in a *Pennisetum* hybrid (Krishnaswamy and Raman 1953). The complete failure of the centromere to divide is an extreme form of these irregularities.

V. ACKNOWLEDGMENTS

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ADDENDUM

Bosemark (1954) has described the behaviour at meiosis of accessory chromosomes in *Festuca pratensis*. These chromosomes are largely heterochromatic. Where a number of univalent accessory chromosomes were present at AI, "difficulties" in their division were occasionally observed. This led to frequent univalent bridges with the chromatid arms apparently directed towards the poles. Groups of such bridges resulted in the formation of restitution nuclei. The general behaviour of these accessory chromosomes resembles that of the univalents in *P. coerulescens* \times *P. minor*, and may well be open to a similar interpretation.

EXPLANATION OF PLATE I

- Figs. 1-9.—*Phalaris coerulescens* \times *P. minor*. Figs. 10 and 11.—*Phalaris tuberosa* \times *P. minor*.
- Fig. 1.—MI with seven bivalents and seven large univalents.
- Fig. 2.—Early AI with six univalents in the equatorial plane.
- Fig. 3.—AI showing five univalents with intercalary regions of the chromatids drawn towards the poles.
- Fig. 4.—Later AI showing the univalents stretched between the poles.
- Fig. 5.—TI with univalent bridges undergoing compression by the formation of the cell wall.
- Fig. 6.—Later TI. The cell wall has nearly broken the univalent bridges.
- Fig. 7.—A II showing one chromosome lying axially under the influence of the neocentromeres.
- Fig. 8.—A II with a five-armed chromosome, and three large and seven small chromosomes in each polar group.
- Fig. 9.—A II with an eight-armed chromosome and a univalent.
- Fig. 10.—A I showing univalent chromosomes moving to the poles under the influence of neocentromeres.
- Fig. 11.—A II with three univalents showing neocentric activity and two showing none.

CENTROMERES IN *PHALARIS*



FUNCTIONAL DIFFERENTIATION IN THE MIDGUT EPITHELIUM OF BLOWFLY LARVAE AS REVEALED BY HISTOCHEMICAL TESTS

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Summary

A series of histochemical tests demonstrates a more striking functional differentiation in the midgut epithelium of *Lucilia cuprina* larvae than is indicated by histological studies. This differentiation is most marked in the middle region of the midgut.

Tests were performed for lipid, phospholipid, glycogen, iron, copper, potassium, ascorbic acid, phosphatases, dehydrogenases, and cytochrome oxidase.

In the anterior and posterior midgut both lipid and glycogen are stored in quantity, and potassium, dehydrogenases, and cytochrome oxidase are present. Both acid and alkaline phosphatases are concentrated in the striated border, but are not conspicuous in the cytoplasm. Iron and copper can be detected only when dietary levels are high.

The mid midgut cells display a complex pattern of functions and five zones can be distinguished. Zone I contains some lipid spheres and stains lightly for copper. In Zone II, two cell types are distinguishable. One contains many lipid spheres, much glycogen, and a cytoplasmic acid phosphatase. The striated border is distinct. The other cell type accumulates copper, iron (but only when dietary copper is high), and possesses cytochrome oxidase and very active dehydrogenases. Glycogen and lipid are absent, acid phosphatase weak, and there is no distinct striated border. In Zone III lipid spheres and copper are present, but iron cannot be detected. Cytochrome oxidase and weak dehydrogenases occur. Zone IV does not react strongly with any of the tests applied. Zone V is rich in iron even on iron-deficient diets and also accumulates copper. Lipid spheres are seldom present except towards the posterior end of the zone. Cytochrome oxidase and dehydrogenases occur. Alkaline phosphatase could not be detected in the mid midgut.

I. INTRODUCTION

The cells of the insect midgut which function in digestion and absorption are usually regarded as being rather uniform histologically. The most notable exception to general uniformity of the midgut epithelium occurs in larval Lepidoptera where columnar and goblet cells are present side by side (Waterhouse 1952).

Some variation in histological structure in different regions of the midgut has been reported in several dipterous larvae and also in some other insects, e.g. Hemiptera, and in groups with symbiont-harbouring mycetomes in the midgut. A degree of functional differentiation in different regions of the midgut

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is suggested by a variation in pH of the digestive juices in different regions, and is demonstrated by the accumulation of materials in the epithelium of some regions but not others.

In many nematocerous Diptera two (and occasionally three) regions of the midgut can be recognized. Thus the cells of the anterior midgut of *Aedes* larvae contain fat globules but no glycogen, whereas the cells of the posterior midgut contain glycogen but no fat (Wigglesworth 1942). Other reports of some degree of functional differentiation include *Ptychoptera* (van Gehuchten 1890), *Anopheles* (Federici 1922), *Chironomus* (Shinoda 1927), *Culex* (Samtleben 1929; de Boissezon 1930a, 1930b, 1930c), and *Simulium* (Pacaud 1950).

In higher Diptera more complex differentiation has been observed. The midgut of *Glossina*, *Lucilia*, and *Drosophila* can be divided into three regions which differ in pH (Hobson 1931; Waterhouse 1940a), histology (Wigglesworth 1929; Hobson 1931; Strasburger 1932), fat content of the cells (Weinland 1908; Hobson 1931), and in the ability to accumulate copper and iron (Waterhouse 1940b, 1945a; Poulson 1950a, 1950b; Poulson and Bowen 1951, 1952; Poulson *et al.* 1952).

In this paper further information is presented on the histological and functional differentiation of the cells of the various regions of the larval midgut of *Lucilia cuprina*. It will be shown that there is a marked functional differentiation between different regions and even between groups of cells in the same region. This functional differentiation is correlated with histological differences although, at first sight, the latter are not striking.

II. METHODS

Almost fully fed third instar larvae of the Australian sheep blowfly *Lucilia cuprina* (Wied.) were used in all experiments. These were generally reared at 30°C under sterile conditions on a standard medium (slightly modified from Lennox 1939) consisting of 87 per cent. egg white, 11.7 per cent. dried baker's yeast, 1 per cent. "Halivol",* and 0.3 per cent. sodium chloride. For certain tests iron, copper, and lipid materials were added to this standard medium. Non-sterile larvae fed on sheep's liver were occasionally used for comparison.

General histological observations on the larval midgut were made both on material fixed in alcoholic Bouin's fluid and stained with Delafield's haematoxylin and eosin, and on material fixed and stained according to Bodian's protargol silver method. Regaud's method was employed for staining mitochondria.

The following histochemical methods were used:

(i) *Lipoid*.—The gut was fixed in Flemming's strong solution for 48-72 hr and washed thoroughly in water. Alternatively it was fixed in 10 per cent. neutral buffered formalin for 18-24 hr and stained for 5-15 min in a saturated solution of Sudan black B in 70 per cent. alcohol. Nile blue was also employed as a stain for certain lipoids (Cain 1947). Baker's (1946) acid haematein test, with controls, was carried out for the detection of phospholipid.

* A vitamin A-rich blend of fish-liver oils (Parke, Davis & Co.).

(ii) *Glycogen*.—Midguts were fixed in alcoholic acetic formalin and stained according to McManus's periodic acid Schiff (PAS) technique (Lillie 1948). Tissues were also freeze-dried,* sectioned, and subsequently fixed briefly in absolute alcohol before staining. Control sections were treated with saliva and then stained. The material which stained red-purple after PAS and which was removed by salivary digestion was considered to be glycogen.

(iii) *Iron*.—Living or formalin-fixed midguts were tested either by the Prussian blue reaction for ferric iron or Turnbull's blue reaction for ferrous iron. Sections were mounted in "Lustrex"† which preserves the colour better than balsam.

(iv) *Copper*.—Equal volumes of fresh 0.1 per cent. aqueous sodium diethyldithiocarbamate and 2 per cent. HCl were added to the freshly dissected midgut (Waterhouse 1945a; Poulson and Bowen 1952). The characteristic yellowish brown coloration of copper carbamate developed to full intensity in a few minutes. Gelatin embedded frozen sections were cut of this material. Alternatively, tissues were fixed in neutral 10 per cent. alcoholic formalin before testing either *in toto* or after frozen sections had been prepared. Some of the tissue copper is in an unionizable form and is not demonstrated by this technique. Saturation of both acid and carbamate solutions with sodium pyrophosphate failed to influence the tissue reactions observed, indicating that it was indeed copper and not iron which was staining. Since pyrophosphate inhibited the Prussian blue reaction there was every reason to believe that it penetrated effectively into the cells.

(v) *Potassium*.—Potassium was precipitated as its cobaltinitrite and made more visible by conversion into cobalt sulphide (Gomori 1952).

(vi) *Ascorbic Acid*.—The method of Sosa (1952) was employed to detect ascorbic acid. His use of an additional washing solution (of sodium sulphite) to remove unreacted silver nitrate more efficiently from the tissues has been stated to improve the specificity of the procedure generally used.

(vii) *Phosphatases*.—The presence of acid and alkaline phosphatases was demonstrated by Gomori's techniques (Gomori 1952). Incubation was carried out for 4 to 24 hr at 37°C with sodium glycerophosphate (B.D.H.) as substrate. Midguts were fixed in cold acetone before staining for acid phosphatase, and in cold 95 per cent. alcohol for alkaline phosphatase. Freeze-dried material after being sectioned, was fixed briefly in absolute alcohol. In addition to sections, whole larval midguts were carried through Gomori's procedure, subsequently dehydrated, embedded, and sectioned. The phosphatases were inactivated in control sections by heat. Sections and whole midguts were also incubated in buffer without substrate.

* Small pieces of gut were frozen rapidly in isopentane cooled with dry ice and methylated spirits. Drying was carried out for 48 hr at -40°C and 0.01-0.02 mm Hg using a W. Edwards & Co. TD4 tissue drier.

† A Monsanto Chemicals Ltd. product.

(viii) *Dehydrogenases*.—The reduction of colourless, water-soluble neo-tetrazolium chloride (NTC) to purple, water-insoluble formazan dye was used to indicate the presence of dehydrogenases. Fresh midguts were incubated according to the method of Seligman and Rutenburg (1951) in a solution of equal parts of 0.1 per cent. NTC, 0.2M sodium succinate, 0.1M phosphate buffer (pH 7.6), and distilled water. Tissues were incubated in covered dishes at room temperature for 2 hr. Tests were also carried out with other substrates or enzyme inhibitors substituted for the succinate of the standard incubation medium. The omission of succinate from the medium did not alter the coloration formed in fresh midguts. However, when the midguts were frozen and thawed, colour was formed only in the presence of succinate or other suitable substrate, presumably because endogenous substrate diffused out of the tissues after thawing. Therefore frozen midguts were used in tests with various substrates.

(ix) *Cytochrome Oxidase*.—Fresh tissues were placed for 1 to 2 min in a shallow container in the following Nadi reagent which was mixed immediately before use: 1 part 0.01M recently recrystallized and freshly made up dimethyl-*p*-phenylene diamine in insect saline, 1 part freshly resublimed 1M α -naphthol in 95 per cent. alcohol which had been diluted 100 times with insect saline just before use, 2 parts 0.1M phosphate buffer (pH 7.4). The tissues were then removed to saline for examination and mounted, if necessary, in 5 per cent. potassium acetate.

III. RESULTS

A. MORPHOLOGY AND HISTOLOGY OF THE MIDGUT

The morphology of the midgut of a well-fed third instar larva is diagrammatically represented in Figure 1. The proventriculus at the anterior end of the midgut is a double walled structure formed by the invagination of the foregut into the midgut. At the base of the proventriculus, four gastric caeca join the midgut; two pairs of malpighian tubules enter the midgut shortly before its posterior end. Although the diameter and length of the midgut is conditioned by the contents of the gut and the contraction of the gut musculature, the different regions of the midgut can be distinguished approximately by their size. As in *Lucilia sericata* larvae (Hobson 1931), the anterior and posterior regions of the midgut are generally thick. The middle region is narrow at either end but greatly distended in the middle where the gut characteristically bends sharply. In the distended bend of the midgut the peritrophic membrane is often coiled. The three regions of the midgut are also distinguished by the pH of their contents (Hobson 1931; Waterhouse 1940a). The anterior and posterior regions are alkaline, pH 7.4-8.0, whereas the middle segment is acid, pH 3.3-3.6 (Waterhouse 1940a).

The midgut is composed of a single layer of large epithelial cells, surrounded by an inner layer of circular muscle and an outer, longitudinal layer of muscle. A peritrophic membrane, produced by cells of the proventriculus, lines the midgut. The free surface of the epithelial cells is characterized by a

striated border. Small cells, which will form the imaginal midgut, occur in clusters along the midgut at the base of the epithelium and are frequently seen in mitotic activity. Regenerative cells are absent and no mitotic division is observed in the large epithelial cells which apparently grow during larval life solely by cell enlargement (see also Trager 1935). The tracheoles of the midgut are closely associated with the muscularis of the midgut and even

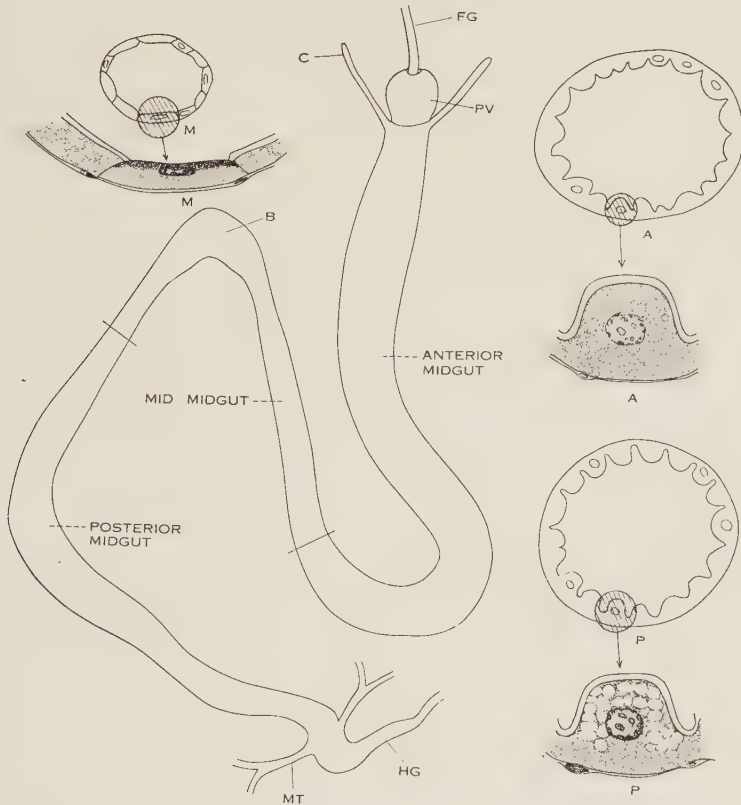


Fig. 1.—Diagram of midgut of *L. cuprina* larva, with camera lucida inserts indicating typical histology of various regions. A, anterior midgut; M, mid midgut (Zone II); P, posterior midgut; B, bend region of mid midgut; C, caeca; FG, foregut; HG, hindgut; MT, malpighian tubules; PV, proventriculus.

penetrate into the epithelial cells. Occasionally, tracheal cells, slightly larger than the imaginal cells, are seen at the base of the epithelial cells. The characteristic histology of the various regions of the midgut epithelium is indicated in the inserts of Figure 1.

(i) *Anterior Midgut*.—Tall, basophilic columnar cells are characteristic of the epithelium of the anterior midgut. The columnar cells of the outer (midgut) wall of the proventriculus have a flat lumen surface and are seldom vacuo-

lated. The remainder of the anterior midgut cells are often vacuolated and are characterized by a free surface which is rounded and extends deeply between the cells (Fig. 1, A). Mitochondria occur as filaments and granules throughout the cytoplasm, but are heavily concentrated at the base of the cells (Plate 2, Fig. 1).

(ii) *Mid Midgut*.—Toward the region of the mid midgut the indentations between the cells are gradually lessened and the cells become low with a flat surface. Two types of cell, vacuolated and non-vacuolated, are intermixed in the anterior portion of the mid midgut (Fig. 1, M). The cells containing round vacuoles in the apical portion of the cytoplasm are also characterized by a distinct striated border, a centrally or basally located nucleus, and cytoplasm uniformly stained with haematoxylin. Mitochondria occur throughout the cytoplasm, but are more abundant at the apex of the cells. On the other hand, the non-vacuolated cells lack a distinct striated border (Plate 2, Fig. 6); the nucleus is apical and the apical cytoplasm stains more heavily with haematoxylin than the basal cytoplasm. Mitochondria are concentrated at the base of the cells (Plate 2, Fig. 2). There are no obvious differences in the degree of tracheation of the vacuolated and non-vacuolated cells. The cells in the distended region of the mid midgut are extremely flattened and of one type only. Differentiation of the cytoplasm or cell border is not easily distinguished in sections. Where the lumen of the mid midgut is again narrow, the cells are cuboidal and all resemble the vacuolated cells anterior to the bend. There are no cells in the mid midgut which are similar to the "calycocytes" figured by Strasburger (1932) in the corresponding region of the midgut of *Drosophila* larvae. However, examination of 5μ sections of the mid midgut of *Drosophila* reveals two cell types. One cell type is large, has an apical nucleus and no distinct striated border, and protrudes into the lumen, so that it overhangs adjacent, smaller cells of the second type. This, in effect, causes the smaller cells which have a distinct striated border, to occupy the base of a cup or flask-shaped depression. These two cell types evidently correspond with those in Zone II of the *Lucilia* mid midgut.

(iii) *Posterior Midgut*.—In the posterior midgut (Fig. 1, P) the indentations between the cells and the rounded free surfaces resemble those of the cells in the anterior midgut. Sometimes discrete rounded vacuoles, similar to those in the anterior midgut, appear in the apical cytoplasm of the posterior midgut cells. Often, however, the entire cytoplasm is highly vacuolated. Mitochondria are scattered throughout the cytoplasm and, in cells with relatively few vacuoles, are seen to be concentrated beneath the striated border (Plate 2, Fig. 3).

B. HISTOCHEMICAL TESTS

(a) *Lipoid*

(i) *Distribution of Osmic-reducing Substances*.—Although osmium tetroxide is now considered unspecific for lipoids (Cain 1950) it is useful for demonstrating the general distribution of fat droplets. When midguts were immersed

in Flemming's solution, blackening commenced rapidly in the cells containing lipoid spheres. Since the gut was later examined in water and without having come into contact with alcohol, it is unlikely that unspecific secondary blackening played an important role in establishing the typical pictures described below.

Larvae fed on standard medium.—The distribution of osmic-blackened substances is illustrated in Figure 2 (a). The anterior midgut is unstained or, at most, stains a light brown. The mid midgut, with the exception of some cells, often stains heavily, but at times may stain lightly or not at all. Extensive blackening occurs in the posterior midgut. Staining is most intense in the middle portion of this region and is frequently weak or absent at its anterior and posterior ends.

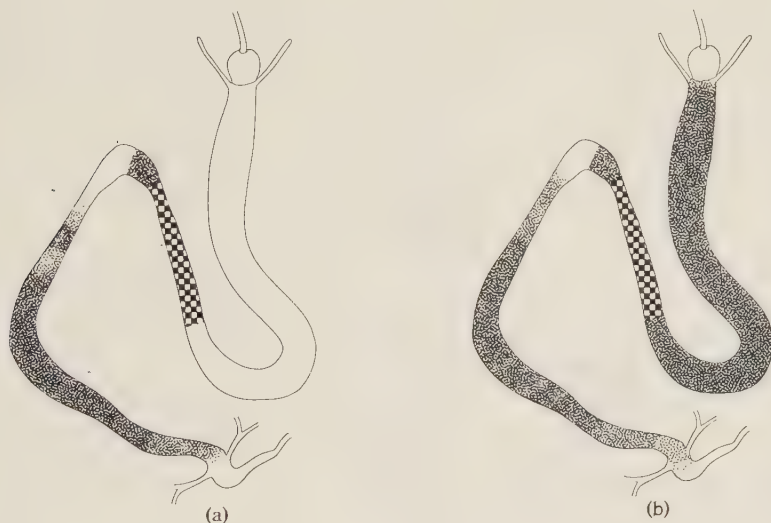


Fig. 2.—Distribution of osmic-reducing lipid in the midgut of *L. cuprina* larvae fed on (a) standard medium, and (b) liver. The intensity of the reaction is indicated by the depth of stippling and the mosaic of mid midgut Zone II by black and white squares.

The distribution of staining in the mid midgut may be described most conveniently by subdividing this region into five zones (Fig. 3 (a); Plate 1, Fig. 5). Staining commences uniformly and rather sharply near the beginning of Zone I and, in surface view, is generally most intense near the periphery of the cells. Passing posteriorly into Zone II the staining increases in intensity. Here, however, interspersed among the stained cells are others which remain unstained. There is a sharp line of demarcation between Zone II and Zone III, and in the latter, almost without exception, all cells reduce osmium tetroxide. Zone IV is well demarcated anteriorly, since its cells are unstained. In Zone V light staining may occur near the periphery of the cells, although this is not common at the anterior end of the zone.

Larvae fed on modified standard medium.—When “Halivol” is omitted from the medium the midgut of most larvae fails to stain, except in the central region of the posterior midgut. When the concentration of “Halivol” is in-

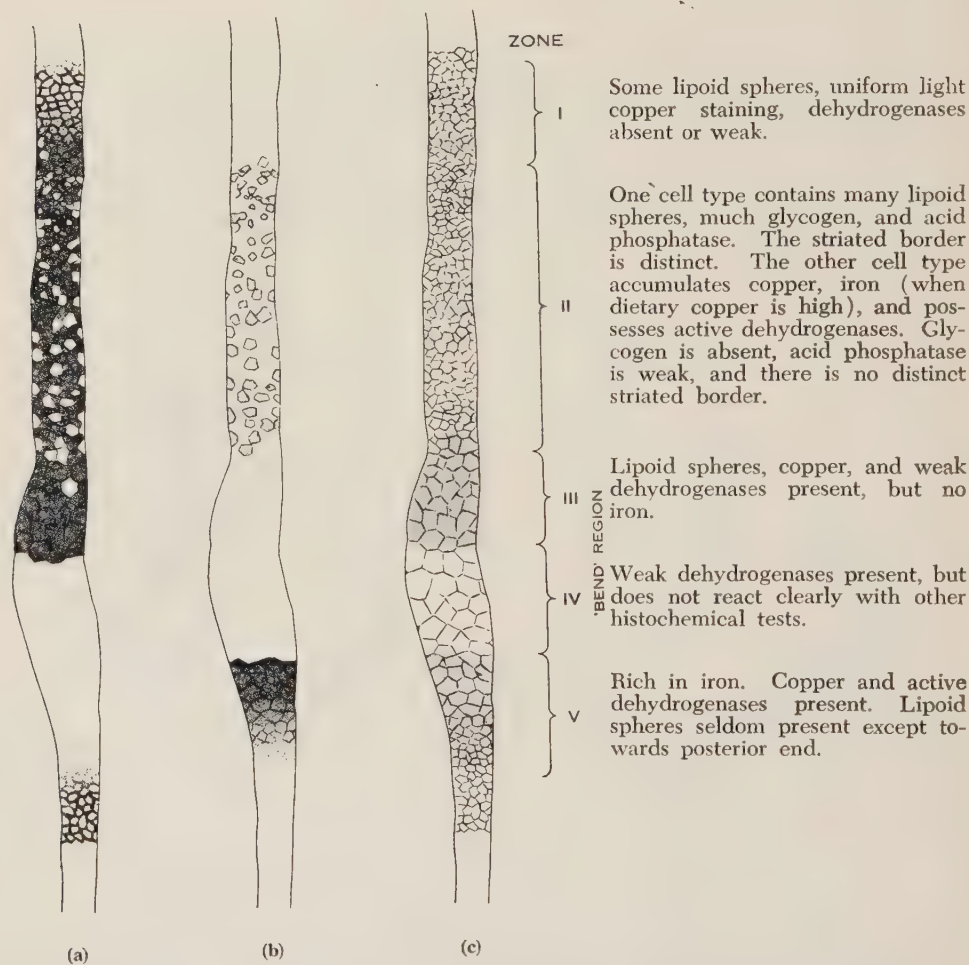


Fig. 3.—Diagram of mid midgut of *L. cuprina* larva, showing distribution of (a) osmic-reducing lipid, (b) ferric iron, and (c) copper. Stippling indicates intensity and distribution of the histochemical reaction. Alkaline phosphatase was not detected in the mid midgut.

creased to 5 per cent., the anterior midgut sometimes stains lightly at its anterior end and the posterior midgut not infrequently stains throughout its entire length. When 10 per cent. “Halivol” is present in the medium, a small amount of staining can be seen throughout the anterior midgut and a general intensification of staining occurs in the other sites of lipid accumulation. Generally all cells stain except (a) those of the caeca (the cells at their bases were sometimes stained), (b) the anterior portion of mid midgut Zone I, (c) the non-staining cells of mid midgut Zone II, and (d) mid midgut Zone IV.

When 500 μ g cholesterol/g is added to the standard medium there is no change in the overall distribution of staining, but there is a distinct tendency for the mid midgut to stain very heavily and for the scattered, unstained cells of Zone II to stand out very distinctly (Plate 1, Fig. 5). However, a series of tests with Schultz's reaction for cholesterol (Lillie 1948) failed to demonstrate the presence of cholesterol in the midgut epithelium, although a positive reaction was noted in the food. Neither glycerol (which had a toxic effect in higher concentrations) nor oleic acid added to the standard medium lacking "Halivol" produces any distinct increase in osmic-reducing substances in the midgut, although there may be a slight intensification of staining in the posterior midgut.

Liver-fed larvae.—The most striking differences between larvae fed on liver (Fig. 2 (b)) and those described above are that the anterior midgut stains heavily and that there tends to be sparsely distributed osmic-reducing spheres in cells which have not stained previously. On the other hand, the mid midgut generally does not stain as heavily and the unstained cells of Zone II still as a rule contain no apparent osmic-reducing material.

Intracellular distribution of osmic-reducing substances.—Osmic reduction appears to be restricted to well-defined lipid spheres which often occur in large numbers in the cells of blackened regions. The nuclei are never stained, nor does there appear to be any osmic reduction in the general cytoplasm or the striated border. When the spheres occur in large numbers they are distributed throughout the cytoplasm; when they are present in smaller numbers they tend to be more numerous in, or even restricted to, the apical half of the cells (Plate 1, Fig. 1). The removal of fat by usual histological procedures gives the cells a vacuolated appearance.

The anterior midgut epithelium of liver-fed larvae is stuffed with blackened spheres, whereas larvae reared on the standard medium contain few, if any, of these. In the mid midgut of larvae fed on standard medium, lipid spheres are typically absent from the non-staining cells of Zone II and present in the stained cells of this zone (Plate 1, Figs. 3 and 4). The contrast in Zone II between the lipophilic cells and the adjacent cells which contain no lipid is also well seen in sections (Plate 1, Fig. 2). Near the anterior end of Zone II some cells which have the general appearance of non-lipoid-accumulating cells do, in fact, sometimes contain relatively small numbers of lipid spheres. These usually occur in one portion of the cell only, namely that adjacent to an adjoining lipophilic cell. Small numbers of lipid spheres may also be present occasionally in the cells of Zone IV where they usually occupy a position near the nucleus. In larvae fed on liver or on standard medium plus 5 or 10 per cent. "Halivol," numerous tiny, osmic-reducing lipid spheres may occur in the apical half of cells which are not normally lipid-accumulating. Because of the position of these tiny spheres and the small proportion of the cytoplasm which they occupy they are not apparent when whole mounts of the osmium tetroxide treated mid midgut are examined.

(ii) *Distribution of Sudan Black B Staining*.—Sudan black B colours only lipid, and all lipoids (except those in the solid state) are coloured by this dye (Cain 1950).

When the formalin-fixed midgut is stained *in toto* with Sudan black B it has a dark blue appearance throughout its length. Examination of pieces of midgut opened out and mounted flat in glycerine jelly reveals that this staining is of two types: (a) all cells contain minute particles of sudanophilic material evenly distributed throughout the cytoplasm to produce a 'ground' coloration; (b) the cells in some regions have, in addition, many intensely sudanophilic spheres. The distribution of these sudanophilic spheres coincides with that of the blackening already described for Flemming-fixed material (Plate 1, Fig. 5).

If formalin-fixed material is extracted at room temperature for several hours with pyridine, acetone, or ether before treatment with Sudan black B, only the 'ground' coloration is observed and this appears to be as intense as in unextracted material. However, extraction with pyridine at 60°C for 18 hr removes all, or almost all, of the materials which colour with Sudan black B.

A characteristic picture of cells from mid midgut Zone II is shown in Plate 1, Figure 6. Cells containing sudanophilic spheres and cells with only a 'ground' coloration are illustrated.

(iii) *Distribution of Nile Blue Staining*.—The oxazine and the oxazone of which this dye is composed stain fatty acids blue and triglycerides red respectively, except when these are in the solid state. Since most elements of a tissue will also stain irrespective of their lipid content it is necessary to define the distribution of lipid by other means (e.g. Sudan black B) (Cain 1947).

When the formalin-fixed midgut is stained *in toto* with Nile blue and differentiated in acetic acid there is a general blue 'ground' coloration in all cells. In addition, where lipid spheres occur, these colour red or purple indicating that they are rich in triglycerides.

(iv) *Phospholipids*.—In the anterior midgut, filaments and granules of dark blue or black material are present throughout the cytoplasm but are concentrated at the base of the cells. Large lipid spheres are stained pale blue. In one larva conspicuous black granules were observed in the cytoplasm of anterior midgut cells.

In the mid midgut the lipophilic cells of Zone II contain pale blue globules as well as fine, dark blue granules at the apex of the cytoplasm. The cells without lipid spheres, on the other hand, exhibit a blue staining at the base of the cell whereas the apex of the cytoplasm is clear. Dark granules and filaments are not as distinct as in the anterior midgut cells.

In the posterior midgut, as in the anterior and mid midgut, large lipid spheres stain pale blue. Dark phospholipid granules are scattered throughout the cytoplasm. In cells which contain few large lipid spheres it is apparent that the small dark granules are concentrated beneath the striated border of the cell.

The intensity of blue staining of the large globules is the same in both medium and liver-fed larvae, although the pale blue globules are more abundant

in the midguts of liver-fed larvae. The distribution and colour of the small granules and filaments is similar in liver and medium-fed larvae. Sections of midguts extracted with pyridine show no pale blue globules, dark granules, or filaments in the cytoplasm.

A pale blue colour does not indicate a strongly positive acid haematein reaction (Baker 1946). Thus the large lipoid spheres which are blackened by osmium tetroxide and Sudan black B seem to contain little if any phospholipid. Phospholipid, as indicated by a dark blue or black acid haematein reaction, is limited to the fine granular and filamentous bodies of the cells. The distribution and size of these small phospholipid bodies so closely parallels that of the mitochondria as to suggest that the latter are staining.

(b) *Glycogen*

The distribution of glycogen in the midgut of larvae fed on standard medium is indicated in Figure 4. In general, the localization of glycogen appears similar to that of lipid. Glycogen is more abundant in the posterior midgut than in the anterior region. In the mid midgut, the cells of Zone II which contain glycogen correspond to the lipophilic cells, whereas glycogen is absent from the nonlipoid cells (Plate 2, Fig. 4). Liver-fed larvae store more glycogen in the midgut than do animals fed on the standard medium. In liver-fed larvae both the anterior and posterior midguts are rich in glycogen and small granules of glycogen are also evident in the lipophilic cells of the mid midgut.

After freeze-drying, glycogen is localized in granules uniformly distributed throughout the cytoplasm or clustered around the lipid vacuoles (Plate 2, Fig. 5). After ordinary fixation and dehydration the granules tend to be clumped at the side or apex of the cells.

The striated border, the peritrophic membrane, and the basement and reticular membranes around the outside of the midgut stain conspicuously after treatment with saliva. Cytoplasmic granules which are resistant to digestion by saliva, occur in cells of the proventriculus, in the anterior and posterior midgut, and in the lipophilic cells of the mid midgut. These granules appear most frequently at the level of the nucleus.

(c) *Iron*

(i) *General Distribution of Ferric and Ferrous Iron*.—As reported by Waterhouse (1940*b*), large amounts of ferric iron and smaller amounts of ferrous iron can be detected in a short band of cells at the anterior end of mid midgut Zone V (Fig. 3 (*b*)) in larvae feeding on the standard medium. This medium contains approximately 13 μg Fe/g (Lennox 1940). No staining is detectable in any other region of the midgut, except occasionally in fully-fed larvae in which the anterior midgut may stain very lightly.

When larvae are fed on iron-enriched media (up to 100 μg Fe/g) the anterior and posterior midguts may stain lightly with the Prussian blue reaction. However, the mid midgut still fails to stain except in the iron-accumulating Zone V, which generally stains more heavily and extensively. This character-

istic distribution can be observed most clearly if larvae are transferred for 90 minutes or more to standard medium before testing. This flushes out the alimentary canal (Waterhouse 1954) and reduces the background colour due to unabsorbed iron in the lumen.

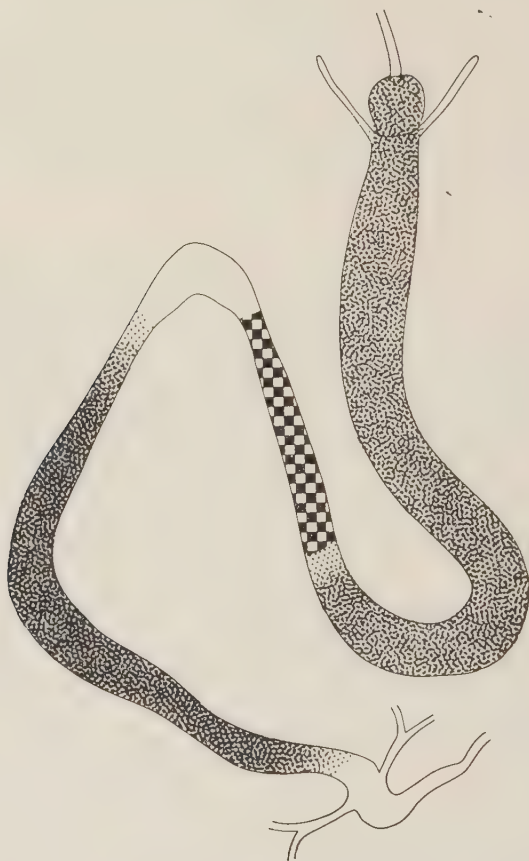


Fig. 4.—Distribution of glycogen in the midgut of *L. cuprina* larvae.

The foregoing applies to larvae fed on media containing about the same level of copper as the standard medium ($4\text{ }\mu\text{g Cu/g}$ (Waterhouse 1945*b*)). When the amount of copper in the medium is increased to about $50\text{ }\mu\text{g/g}$ or more and irrespective of whether or not more iron is added to the medium, a mosaic of cells in mid midgut Zone II also stains intensely for ferric and ferrous iron (Fig. 3 (*b*); Plate 2, Fig. 7). It can be seen in sections of Zone II or in pieces opened out and mounted flat that these iron-rich cells contain few or no lipid spheres. The iron-rich cells are clearly distinguished from adjacent non-staining cells which are packed with lipid spheres. Iron can still be detected in these scattered cells 7 hr after larvae have been transferred to standard medium, indicating that it is not merely present in a mobile form awaiting excretion or uptake into the haemolymph. The accumulation of stainable iron

in Zone II of the midgut is apparently conditioned not only by the availability of iron in the food, but also by the level of copper in the diet, since cells of Zone II do not stain for iron in larvae fed on medium rich in iron alone ($100\text{ }\mu\text{g Fe/g}$). Furthermore they stain only lightly or not at all on a diet containing as much as $20\text{ }\mu\text{g Fe/g}$ and only $25\text{ }\mu\text{g Cu/g}$. The relatively small amount of iron present as an impurity in the added copper does not, therefore, appear to be of any importance.

(ii) *Intracellular Distribution of Detectable Iron*.—There is a strong tendency for iron staining to occur mainly or entirely in the apical half of the cell. Nuclei do not stain. Much of the colour is due to small, blue cytoplasmic granules. The majority of these are less than $1.5\text{ }\mu$ in diameter. There also appears to be some lighter, more general, and not obviously granular staining of the cytoplasm, although this may be mainly due to granules which lie out of focus. On the other hand, in larvae fed on iron-rich diets where staining occurs in cells other than those which accumulate massive deposits of iron, the colour is diffuse and does not appear to be associated with granules.

In those cells which accumulate iron, stained granules sometimes occur only in a narrow band immediately below the striated border. At other times granules may occupy a more central zone in the apical half of the cell or may exist in both positions. Many unstained refractile granules appear in the basal half of the iron-rich cells, particularly in Zone II. There is good evidence from living material that the stored iron which can be detected in mid midgut Zone V is indeed present mainly in the form of granules. This zone has a light yellowish brown appearance when the Prussian blue test subsequently demonstrates abundant iron to be present. Examination of this region cut open and mounted flat in saline reveals that the yellowish brown coloration is due to accumulations of numerous small, light brown granules. Similar granules are not observed in cells of other regions which give no iron reaction. It is assumed that the iron is present in these granules as a haemosiderin- or ferritin-like complex. Evidence favours the former which, unlike ferritin, is histochemically detectable. Furthermore, ferritin crystals were not produced following incubation with 10 per cent. cadmium sulphate.

(d) Copper

(i) *General Distribution of Detectable Copper*.—There is some variability in the amount of stainable copper from larva to larva in the same culture, and an even greater variation between cultures set up on different occasions. Some larvae stain quite heavily and distinctly, others stain lightly.

Copper-staining with diethyldithiocarbamate in larvae fed on the standard medium occurs only in the mid midgut, where copper may be detected in all zones except Zone IV (Fig. 3 (c)). When staining occurs in Zone I it is light and uniform. Zone II consists of some cells which stain heavily and others which stain comparatively lightly or not at all (Plate 3, Fig. 3). Zones III and V are generally uniformly and fairly heavily stained. If, after staining, Zone II is slit longitudinally, the contents removed, and the epithelium mounted, it can be seen that the intense stain for copper occurs in cells which do not con-

tain lipid spheres, although some of the non-lipoid cells may only stain lightly. The cuprophilic* cells of Zone II are those in which iron can be detected on diets rich in copper. It is necessary to correct an earlier statement (Waterhouse 1945a) inferring that the cuprophilic cells also contain osmiophilic lipid. Confusion arises from the statement regarding the relative sizes of the two types of cell. At the anterior end of Zone III both the lipophilic and cuprophilic cells are approximately the same size. However, towards the posterior end of this zone, the cuprophilic cells are often larger than the lipophilic cells.

When the concentration of copper in the medium is increased (up to 100 μg Cu/g) a positive carbamate reaction becomes more general. The anterior and posterior midguts are unstained except at high concentrations of copper and then they stain only weakly. As the concentration of dietary copper rises, staining is first visible in the final third of the posterior midgut and may also extend for a short distance posterior to the entrance of the malpighian tubules. When more copper is ingested, the entire posterior midgut may stain. Staining becomes more general in the mid midgut as the copper concentration increases, although the previously unstained cells of Zones II and IV never stain heavily. These observations confirm and extend those of Waterhouse (1945a).

(ii) *Examination with Ultraviolet Light*.—If the midgut of larvae fed on standard medium containing at least 50 μg Cu/g is examined under ultraviolet light, mid midgut Zones II, III, and V show a distinct orange-red fluorescence in contrast with the pale blue of other regions. With increasing concentrations of dietary copper, this orange-red fluorescence becomes more distinct and may extend into other regions of the midgut. When the fluorescence is intense two cell types clearly occur in Zone II. Larvae fed on the standard medium without added copper or on some samples of sheep's liver do not show a visible fluorescence. Other batches of liver-fed larvae may fluoresce distinctly, particularly when copper is added to the ground liver on which they are fed. The liver may itself exhibit a similar fluorescence. The fluorescence of the midgut is intensified on drying and persists for several weeks in dried preparations. It is abolished by treatment with carbamate, cyanide, or cupric sulphate. These findings closely parallel those of Poulson and Bowen (1952) for *Drosophila* larvae.

(iii) *Intracellular Distribution*.—In frozen sections of mid midgut Zones II and V it can be seen that copper diethyldithiocarbamate is largely restricted to the apical half of the cell and that the nuclei fail to stain. When the cells are heavily stained, the copper carbamate is associated with cytoplasmic granules of varying size and there is also a generalized cytoplasmic staining not apparently associated with granules. The copper granules are believed by Poulson and Bowen (1952) to be mitochondria and possibly Golgi material. Since, in mid midgut Zone II, the mitochondria are concentrated at the base of the copper cells and the often large copper granules at the apex, it is improbable that there is any correlation between the two in *L. cuprina*.

In the posterior midgut of larvae fed on high concentrations of copper, the copper carbamate is initially present in a diffuse form throughout the cyto-

* This term is used for cells rich in ionizable cupric or cuprous salts.

plasm. After a short time the general cytoplasm becomes colourless and crystals of copper carbamate form, mainly on the nuclear membrane. There is no apparent association here with cytoplasmic granules. These observations parallel those of Poulson and Bowen (1952) in *Drosophila* larvae.

(e) Potassium

The method employed for detecting potassium is said to be adequately specific if sufficient time is given for any ammonium cobaltinitrite formed to be washed out of the tissues by ice-cold water.

On the basis of the test employed, potassium is present in readily detectable amounts in the anterior and posterior midguts, but is apparently absent or present in relatively low concentration in the mid midgut. There are no striking differences between the various mid midgut zones. The proventriculus and the anterior fifth of the anterior midgut are generally fairly heavily stained, as is also the anterior quarter of the posterior midgut. There are often other zones of the anterior and posterior midgut which may stain quite heavily, but there does not appear to be any constancy in their position. Larvae fed on liver or on standard medium enriched with potassium chloride provide the same picture of potassium distribution, but with a heavier and somewhat more general staining of the tissues. In sections, the staining is seen to be due to massed granules, ordinarily restricted to the basal quarter of the cell and to the muscular layers.

(f) Ascorbic Acid

No ascorbic acid can be detected in any region of the midgut of larvae fed on standard medium. Most liver-fed larvae also fail to react, although a faint precipitate of reduced silver can occasionally be detected in the mid midgut. As Day (1949) recorded ascorbic acid granules scattered throughout the midgut cells of *L. cuprina* (presumably fed on liver) his technique was employed, but also with negative results. He obtained clear evidence that the amount of ascorbic acid in the tissues was greatly influenced by its level in the diet. Ascorbic acid was therefore added to the standard medium (approximately 10 $\mu\text{g/g}$ medium) before or after autoclaving. The resulting larvae were subsequently tested both before and after the ascorbic acid medium had been flushed out of the digestive tract by transfer to control medium. As a rule no ascorbic acid could be detected in the midgut epithelium although odd granules could occasionally be seen. On the other hand, the ascorbic acid-enriched food in the digestive tract gave a strong positive reaction.

(g) Phosphatases

The distribution of phosphatases is indicated in Figures 5(a) and 5(b). Alkaline phosphatase is concentrated in the striated border of the anterior and posterior midgut. Nuclei stain weakly and cytoplasm not at all. The mid midgut shows little or no alkaline phosphatase activity in either sectioned or whole preparations.

Acid phosphatase is likewise localized in the striated border of the anterior and posterior midgut but occurs also in all midgut nuclei. The mid midgut does not show enzyme activity in sectioned material, but *in toto* preparations exhibit a mosaic of phosphatase activity in Zone II (Plate 3, Fig. 1). Examination of sections made subsequent to staining reveals that acid phosphatase is more active in the lipophilic cells (where it occurs mainly at the base of the cytoplasm) than in the cuprophilic cells. In Zones III and IV, only the nuclei show acid phosphatase activity.

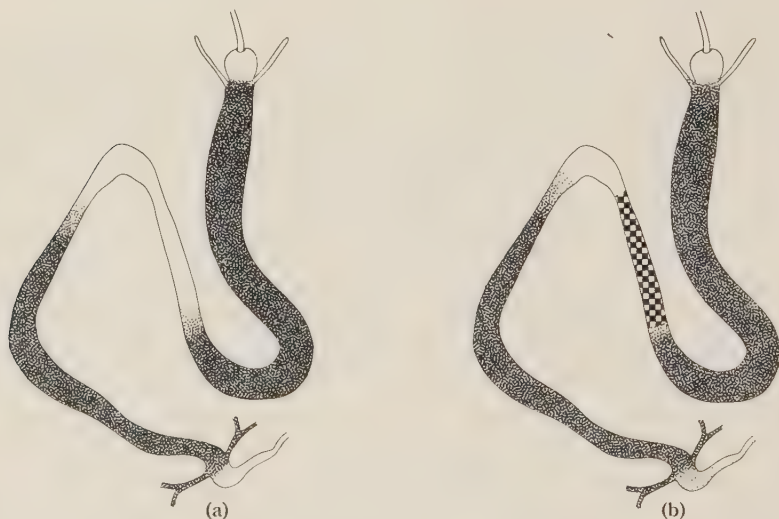


Fig. 5.—Distribution of phosphatases in the midgut of *L. cuprina* larvae.
(a) alkaline phosphatase, (b) acid phosphatase.

A precipitate which formed in both the acid and alkaline media during the incubation of whole midguts was taken to indicate diffusion of enzyme or reaction products. It is probable that diffusion and loss of enzyme activity would be even greater in sectioned material. Thus the acid phosphatase which can be demonstrated in whole mid midgut preparations may have been lost by diffusion during sectioning and staining of the tissue. The enzyme activity of freeze-dried sections is not obviously greater than that of ordinary sections, although there appears to have been less diffusion in the freeze-dried material. Thus alkaline phosphatase is limited to the striated borders in freeze-dried sections (Plate 3, Fig. 4), whereas the nucleus, portions of the cytoplasm, and the contents of the lumen may also be blackened in ordinary sections (Plate 3, Fig. 2).

(h) Dehydrogenases

The distribution of dehydrogenases is indicated in Figure 6. The anterior and posterior midgut stain deeply with purple formazan dye. In the mid midgut the distribution of formazan is similar to that of copper (Fig. 3(c); Plate 3, Fig. 8). The striking dark purple cells of Zone II correspond to the cuprophilic

cells (Plate 3, Fig. 6). The lipophilic cells of Zone II stain only lightly as do Zones I, III, and IV. Zone V is heavily stained. The posterior midgut is often irregularly stained. Some groups of cells among the purple ones are colourless. Other groups of cells, where the purple formazan is dissolved in the lipoid spheres, appear red.

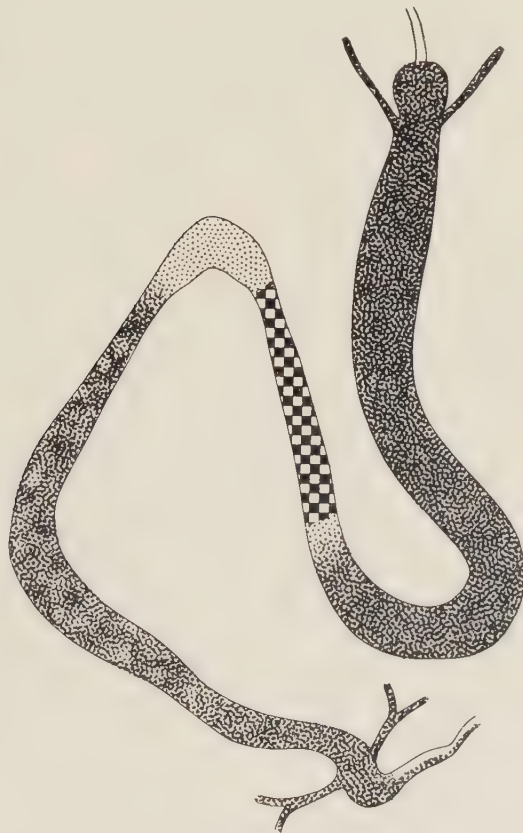


Fig. 6.—Distribution of dehydrogenases in the midgut of *L. cuprina* larvae.

The intracellular localization of the formazan was examined in $10\ \mu$ frozen sections of the midgut cut after incubation and formalin fixation. Granules of purple dye are deposited throughout the cytoplasm of the anterior and posterior midgut cells (Plate 3, Fig. 7). Lipoid globules stain pink or red and are often surrounded by dark purple formazan. The nucleus and striated border are unstained. In the cuprophilic cells of mid midgut Zone II, formazan is concentrated at the apex of the cytoplasm (Plate 3, Fig. 5).

Frozen midguts incubated in buffered NTC solution reduce NTC when succinate, lactate, or glycerophosphate ($5 \times 10^{-2}\text{M}$, final concentration) are present in the incubation medium. Malate, fumarate, and citrate (at the same concentration), and xanthine at $6.6 \times 10^{-4}\text{M}$ are not effective substrates.

Monoiodoacetate partially inhibits the deposition of formazan at $2.5 \times 10^{-2}M$ final concentration and completely inhibits the reaction at $5 \times 10^{-2}M$ concentration. Malonate at $10^{-1}M$ concentration partially inhibits the reduction of NTC.

(i) Cytochrome Oxidase

When the midgut is immersed in a thin layer of freshly prepared Nadi reagent the first coloration is almost always seen in the caeca and in those cells of mid midgut Zone II which accumulate copper and reduce tetrazolium salts. Subsequently, the remainder of the midgut, with the exception of Zone IV, commences to take on a blue coloration which increases in intensity with further exposure to the reagents. Zone IV colours comparatively slowly. A noticeable feature of preparations which have stood in the reagent for several minutes is the purplish coloration of lipid spheres wherever they occur in the midgut epithelium. This is due to a non-specific reaction, the solution of indophenol blue in lipid material.

When the midgut is soaked for 10 min in 0.003M sodium azide and transferred to the Nadi reagent plus azide, the rate of colour development is greatly retarded. The mid midgut stains first and the anterior and posterior midguts stain lightly and slowly. It is noteworthy that the cuprophilic cells of Zone II show little coloration compared with the adjacent lipophilic cells. Azide at this concentration is a partial inhibitor of cytochrome oxidase, although it does not inhibit the autoxidation of the Nadi reagent. Guts which had been immersed in near boiling water for 2 min showed little coloration after immersion in the Nadi reagent. It appears therefore that cytochrome oxidase is generally distributed throughout the midgut and is particularly active in the cuprophilic cells and in the midgut caeca.

Examination of mounted preparations reveals that the blue coloration of the cuprophilic cells is due to numerous small granules or spheres, located mainly in the apical region of the cells. These are few in number or absent in the adjacent lipophilic cells.

C. REDOX POTENTIAL OF THE DIGESTIVE JUICES

When actively feeding larvae are transferred for several hours to casein moistened with solutions of appropriate indicators, no evidence of dye reduction in the midgut is observed after ingestion of potassium indigo tri- or tetra-sulphonate, neotetrazolium chloride, thionine, or 1-naphthol-2-sodium sulphonate indophenol. This indicates that the midgut digestive juices are at least mildly oxidizing. The anterior midgut epithelium accumulates a small amount of thionine and 1-naphthol-2-sodium sulphonate indophenol in their oxidized form; elsewhere in the epithelium no colour is visible even after the addition of oxidizing agents.

D. COMPARISON WITH OTHER DIPTEROUS LARVAE

It is relevant to consider briefly whether the results obtained for *L. cuprina* larvae are peculiar to this species or whether they apply also to larvae of other higher Diptera. A similar mosaic in the mid midgut is present in a number of

species of blowfly larvae other than *L. cuprina*, namely *L. sericata* (Meig.), *Calliphora augur* (F.), *C. stygia* (F.), *Chrysomyia rufifacies* (Macq.), and in the larvae of the housefly *Musca domestica* L. The work of Poulson and his colleagues indicates that, like *L. cuprina*, many species of *Drosophila* accumulate iron and copper in the mid midgut, although no mention is made in these papers of a mosaic of cells of different function in this region. By means of the tetrazolium and Sudan black B tests it is possible to demonstrate that two types of cell occur side by side in the mid midgut of *Drosophila melanogaster* Meig. larvae. However, inadequate information is available to say whether or not these two types correspond functionally with the two types in *L. cuprina*.

IV. DISCUSSION

Table 1 summarizes the results of the various tests performed. Insufficient data are available to enable clear functional correlations to be established between enzyme systems which are present and cellular function even in the mid midgut. It is evident, however, that the blowfly larval midgut with its large cells which neither divide nor become 'worn out' provides ideal experimental material for a detailed study of these correlations.

Control of iron absorption in *L. cuprina* larvae appears to be broadly similar to that in vertebrates where the intestinal mucosa regulates the amount absorbed and little of the iron taken up is excreted. In *L. cuprina*, iron is accumulated from diets poor in this element by certain cells in the mid midgut epithelium. Although an increase in the iron content of the food causes some additional iron to be absorbed, the amount taken up is far from proportional to the increased dietary concentration, and no ionized iron can be detected in the malpighian tubules (Lennox 1940; Waterhouse 1940*b*). In *L. cuprina*, the histochemically detectable iron is typically associated with numerous granules of brown pigment which bears some resemblance to hemosiderin, although a ferritin-like complex may also be present. In vertebrates ferritin is believed to be intimately concerned with the mucosal block which prevents uptake of more than a limited amount of iron. However, some mammals (e.g. sheep and cattle) do not give a positive cadmium sulphate test for ferritin, so that the negative result for *L. cuprina* is inconclusive. Granick (1953) has suggested that a prime function of gastric acidity in vertebrates is to provide conditions under which ionized iron is capable of existing long enough to render absorption possible. Although there is no evidence for insects, as there is for vertebrates, that iron can only be absorbed in the ferrous state it is an interesting parallel that the iron-accumulating cells of *L. cuprina* occur in the acid region of the midgut.

In *L. cuprina* larvae, as in vertebrates, copper appears to be lost continually by excretion (it is abundant in the malpighian tubules) and must be replenished from the diet. Nevertheless, there is some degree of regulation of the copper uptake by the body. Although the amount of copper absorbed increases with increasing dietary concentration, the increase is not proportional to the amount ingested (Waterhouse 1945*b*) and, furthermore, there is evidently a mechanism for accumulating and storing copper from diets poor in copper.

In higher animals the absorption of iron has often been reported to be unaffected by the copper level in the diet, although it is well established that copper has an important influence on the utilization of iron (Marston 1952). However, recent careful work with rats (Chase *et al.* 1952) and pigs (Gubler *et al.* 1952) indicates that the amount of iron accumulated is positively corre-

TABLE 1
SUMMARY OF CHARACTERISTIC RESULTS WITH VARIOUS HISTOCHEMICAL TESTS ON THE
L. CUPRINA MIDGUT

A blank or dash indicates a negative reaction

Histochemical Test	Midgut							
	Anterior	Mid Zone:						Posterior
		I	II		III	IV	V	
			A*	B†				
Lipoid spheres	— to +++	+	+++		+++		±	+++
Glycogen	++	+	+++				±	+++
Iron				— to ++			+++	
Copper		±		++	±		++	
Potassium	+			++				+
Ascorbic acid								
Phosphatase								
Acid	+++ in striated border		++					+++ in striated border
Alkaline	+++ in striated border							+++ in striated border
Dehydrogenases	+++	±	±	+++	±	±	+	+++
Cytochrome oxidase	++	+		+++	+		+	++

* Lipophilic cells.

† Cuprophilic cells.

lated with the level, within certain limits, of the copper in the tissues, and that the latter is in turn related to the amount of copper in the diet. In *Drosophila* larvae, as dietary copper increases, less and less ionizable iron is found in the cytoplasm (Poulson and Bowen 1952). On the other hand, in *Lucilia* larvae the situation is different at least with respect to the cuprophilic cells in mid midgut Zone II. Available evidence indicates that these cells only accumulate ionized iron in detectable concentration when the level of dietary copper is relatively high.

There is a remarkable coincidence between the distribution of copper in the midgut and a compound exhibiting orange-red fluorescence. However, copper could be demonstrated by the carbamate reaction at dietary levels lower than those at which fluorescence was apparent. Poulson and Bowen (1952) have reported the parallel occurrence of a similar fluorescence and of copper in the midgut of *Drosophila* larvae, and in various tissues of other invertebrates. Although copper porphyrins which fluoresce in solution are apparently unknown, these authors state that some do fluoresce in the solid state. If the orange-red fluorescence is indeed due to a copper compound, then their postulate is not unreasonable that much of this compound is present in intracellular granules and is, therefore, essentially in a solid state.

Very little is known concerning the absorption of carbohydrates by the insect gut, although enzymes are present which are capable of degrading those carbohydrates which can be utilized as food (Waterhouse and Day 1953). Nutritional studies indicate that polysaccharides must be hydrolyzed to their component monosaccharides before absorption and it is not improbable that phosphorylation of sugars is involved in the latter process. Certainly hexose phosphates are rapidly absorbed from the midgut lumen in *Periplaneta*. Furthermore, the presence of zinc inhibits the uptake of glucose-6-phosphate and fructose-6-phosphate, which was taken to indicate the presence in the gut of phosphoglucomutase (converting glucose-6- to glucose-1-phosphate) (Hassett 1951). During glycogen formation in mammals phosphorylated hexoses must subsequently be converted to glucose-1-phosphate and then dephosphorylated and polymerized (Sourkes 1953). It is, perhaps, worthy of note that acid phosphatase is far more active in the cells of mid midgut Zone II which store glycogen than in the adjacent copper-accumulating cells. Morton (1953) has shown that phosphatases catalyze both dephosphorylation and transphosphorylation. Whether or not the acid phosphatase in these Zone II cells plays any part either in dephosphorylation leading to glycogen formation or in the subsequent degradation of glycogen is unknown.

In the *L. cuprina* midgut, freeze-dried preparations indicate that alkaline phosphatase is restricted to the striated borders of the cells and does not occur in the nuclei (as in some animals (Chèvremont and Firket 1953)) or in the general cytoplasm, although it appears in these locations when more conventional methods are used.

The very high activity of dehydrogenases and of cytochrome oxidase in the copper cells of mid midgut Zone II may indicate that these cells have a very active oxidative metabolism. Electrons are transferred from succinate to oxygen via the succinic dehydrogenase and cytochrome oxidase systems. Succinic dehydrogenase is generally regarded as being predominantly bound to mitochondria (Holter 1954). However, there is a rather poor correlation between the distribution of mitochondria and of formazan granules in Zone II. This may indicate that dehydrogenases other than succinic are very active in these cells or that the dehydrogenase activity is mainly associated with insoluble granules other than those demonstrated by the mitochondrial staining technique employed.

V. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1-3

All photographs are of larvae fed on standard medium except where noted.

PLATE 1

Lipoid

- Fig. 1.—Flemming's fixation. 10μ section of posterior midgut, showing lipid spheres aggregated in apical half of cell.
- Fig. 2.—Flemming's fixation. 10μ section mid midgut Zone II, showing distribution of lipid spheres (black) in one cell type and their absence in the other cell type which has an apical nucleus.
- Fig. 3.—Flemming's fixation. Mosaic of darkly staining cells containing lipid spheres and light cells without lipid spheres. Spread of mid midgut near posterior end of Zone II.
- Fig. 4.—Flemming's fixation. Lipophilic cell in spread of mid midgut Zone II and portions of adjacent cells without lipid.
- Fig. 5.—Flemming's fixation. Larva fed on standard medium plus $500\mu\text{g}$ cholesterol/g. Whole mount of mid midgut Zones I (top) to III (bottom), illustrating uniform peripheral staining in Zone I, mosaic staining in Zone II, and uniform heavy staining in Zone III.
- Fig. 6.—Sudan black B after formalin fixation. Liver-fed larva. Mid midgut Zone II, cut open and mounted flat, illustrating the ground staining of both cell types and the accumulating of lipid spheres in one type only.

PLATE 2

Mitochondria, Glycogen, and Iron

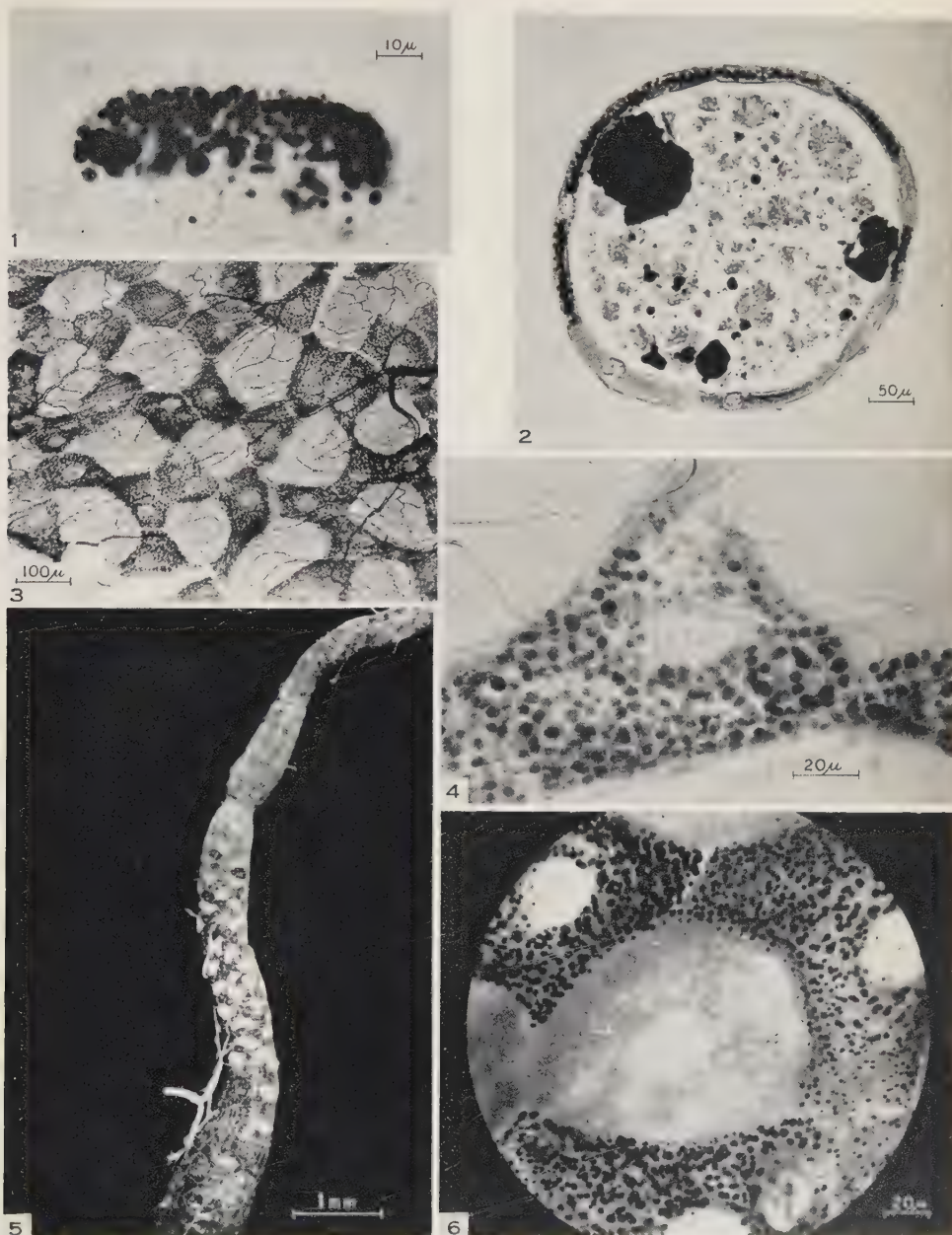
- Fig. 1.—Regaud's method for mitochondria. 3μ section of anterior midgut showing concentration of mitochondrial rods at base of cells and less frequent occurrence elsewhere.
- Fig. 2.—Regaud's method for mitochondria. 3μ section of non-vacuolated cell from mid midgut Zone II, showing the basal concentration of mitochondria.
- Fig. 3.—Regaud's method for mitochondria. 3μ section of posterior midgut. Mitochondria occur throughout the cell, but are most numerous near the striated border.
- Fig. 4.—McManus's periodic acid Schiff (PAS) after freeze-drying and brief acetic-formol-alcohol fixation. Liver-fed larva. 7μ section of mid midgut Zone II, showing dark stain for glycogen in lipophilic cells interspersed with unstained cuprophilic cells.
- Fig. 5.—As for Fig. 4. Higher magnification of a glycogen-containing cell showing granules of glycogen surrounding lipid vacuoles.
- Fig. 6.—PAS stain after salivary digestion. Acetic-formol-alcohol fixation. 7μ section of mid midgut Zone II. Glycogen-containing cell on right with heavily stained, striated border. Copper cell on left with inconspicuous border. The diagonal junction of these cells is typical.
- Fig. 7.—Larva fed on standard medium plus $50\mu\text{g}$ Cu/g. Prussian blue reaction on fresh mid midgut Zones II (top) to V (bottom left). Zones III and IV are the short unstained regions on either side of the bend.

PLATE 3

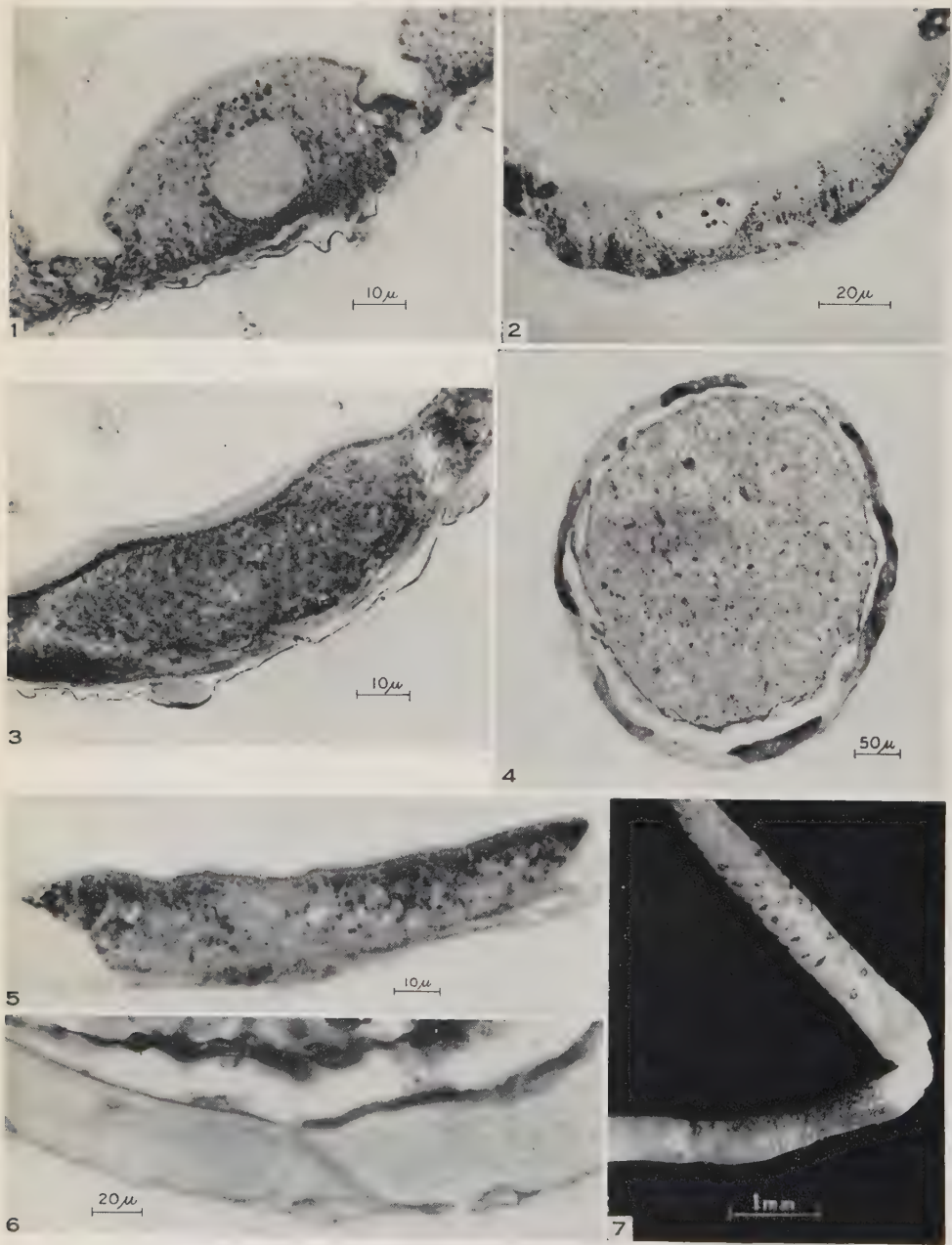
Phosphatases, Copper, and Dehydrogenases

- Fig. 1.—Gomori's acid phosphatase. Acetone fixation. Spread of mid midgut Zone II with lipid cells darkly stained.
- Fig. 2.—Gomori's alkaline phosphatase. 95 per cent. alcohol fixation. 7μ cross section of anterior midgut. Note stained nuclei and stain in lumen of gut. Cytoplasmic staining is not shown in this photograph.

HISTOCHEMISTRY OF INSECT MIDGUT



HISTOCHEMISTRY OF INSECT MIDGUT



HISTOCHEMISTRY OF INSECT MIDGUT



- Fig. 3.—Diethyldithiocarbamate—HCl test. Leitz, Heine condenser, bright field. Spread of mid midgut Zone II showing copper cells (darkly stained) and lipid cells (light). The outline of the lipid spheres in the latter can be seen.
- Fig. 4.—Gomori's alkaline phosphatase. Freeze-dry. 7 μ cross section of anterior midgut. Note sharp staining of striated border and absence of stain in the nucleus.
- Fig. 5.—Dehydrogenases demonstrated with neotetrazolium chloride (NTC). Formalin fixation. 10 μ gelatine cross section of mid midgut Zone II showing aggregation of formazan (reduced NTC) granules in the apex of the two copper cells and absence of dehydrogenase activity in the central lipid cell.
- Fig. 6.—Dehydrogenases demonstrated with NTC. Formalin fixation. Spread of mid midgut Zone II showing intense dehydrogenase activity, revealed by formazan granules, in the copper cell. Adjacent lipid cells are lightly stained.
- Fig. 7.—Dehydrogenase demonstrated with NTC. Formalin fixation. 10 μ gelatine cross section of anterior midgut. Formazan (reduced NTC) is deposited in granules throughout the cytoplasm; nucleus and striated border unstained.
- Fig. 8.—Dehydrogenases demonstrated with NTC. Formalin fixation. Whole mount of mid midgut Zones I (top) to IV (bottom) showing dehydrogenase activity only in the copper cells of Zone II.

THE ELASTIC ANISOTROPY OF KERATINOUS SOLIDS

II. THE RIGIDITY CONSTANTS OF RAM'S HORN

By K. RACHEL MAKINSON*

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Summary

The rigidity constants of ram's horn have been determined by using a pulse technique to measure the velocities of propagation along the principal axes of transverse elastic waves of frequency 4 Mc/s. The results show that the conclusion, which was drawn previously from measurement of the dilatational constants, that ram's horn is transversely isotropic about the radial direction, is approximately though not exactly correct. The type of anisotropy and the relative magnitudes of the various elastic constants are directly correlated with the histological structure of the horn, which under the conditions of the measurements is more important than the molecular structure in determining the nature of the elastic anisotropy.

Denoting the radial, circumferential, and growth directions of the horn by r , θ , and z respectively, the rigidity constants for shear in the planes θz , zr , and $r\theta$ are 2.4_6 , 2.1_3 , and $2.1_6 \times 10^{10}$ dyn cm $^{-2}$. The elastic constant which specifies the relation between the tensile stress in the θ direction and the extension in the z direction is about 5.7×10^{10} dyn cm $^{-2}$.

I. INTRODUCTION

In Part I of this series (Makinson 1954) values were reported for the dilatational elastic constants of various forms of keratin at 5 Mc/s; these were obtained by measurement of the velocities of ultrasonic waves in keratin, using a total reflection method with continuous waves. Theoretically, it should be possible to calculate the velocities of shear waves, and hence the rigidity constants, from the data obtained in the course of these experiments. In practice, only very approximate values could be obtained by these calculations, because the results were extremely sensitive to the initial data. Most of the measurements reported here were therefore made by a different method, using a pulse technique.

Throughout this work, the classical theory of elasticity for small strains has been used. Although this theory is not strictly applicable to a material of so complex a structure as keratin, it is adequate to describe the results within the accuracy of the measurements so far made.

II. NOTATION FOR THE ELASTIC CONSTANTS

It is necessary to describe briefly the usual crystallographic representation of the elastic constants of an anisotropic solid, in order to show clearly which constants have been measured and what assumptions or approximations were involved in their measurement. A fuller description is given by Mason (1950).

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In a Cartesian coordinate system with axes x_1 , x_2 , and x_3 , the behaviour of an anisotropic solid is described by 36 constants

$$\left. \begin{array}{cccccc} c_{11} & c_{12} & c_{13} & c_{14} & c_{15} & c_{16} \\ c_{21} & c_{22} & c_{23} & c_{24} & c_{25} & c_{26} \\ c_{31} & c_{32} & c_{33} & c_{34} & c_{35} & c_{36} \\ c_{41} & c_{42} & c_{43} & c_{44} & c_{45} & c_{46} \\ c_{51} & c_{52} & c_{53} & c_{54} & c_{55} & c_{56} \\ c_{61} & c_{62} & c_{63} & c_{64} & c_{65} & c_{66} \end{array} \right\}, \dots (1)$$

between which the relations $c_{ij} = c_{ji}$ hold, so that there are at most only 21 different constants. In solids which have sufficiently high crystallographic symmetry with respect to the axes, the matrix (1) is simplified by the disappearance of the shear cross-constants c_{ij} , $i \neq j$, i and/or $j > 3$. This is at least approximately the case for ram's horn, and these constants will throughout this paper be equated to zero.

The constants c_{ii} with $i = 1, 2$, or 3 (i.e. c_{11} , c_{22} , and c_{33}) represent the tensile stress in the direction x_i per unit extension in the same direction when all other strains are zero. They determine the velocity of propagation, in an infinite bulk of the solid, of longitudinal elastic waves travelling along the corresponding axes. These constants were measured in the work described in Part I, where they were written c_{rr} , $c_{\theta\theta}$, and c_{zz} respectively.

The constants c_{ii} with $i = 4, 5$, or 6 represent the shear stress in the plane x_2x_3 , x_3x_1 , or x_1x_2 respectively, per unit shear strain in the same plane, all other strains being zero, i.e. they are the principal moduli of rigidity of the solid; they determine the velocities of shear waves propagated along the principal axes. These are the constants which have been measured in the work described here.

The constants c_{ij} with $i \neq j$ and $i, j = 1, 2$, or 3 are related to the Poisson ratios of the solid; they represent the tensile stress in the x_i direction per unit extension in the x_j direction, all other strains being zero. One of them is evaluated in this paper.

When ram's horn is being considered, the axes x_1 , x_2 , and x_3 will be identified with the axes r , θ , and z , respectively, of Part I. The direction r is the radial direction of the horn, θ is the circumferential direction, and z the grain or growth direction. These are Cartesian, not cylindrical polar, axes.

A type of symmetry which will frequently be referred to in connection with ram's horn is transverse isotropy about x_1 , i.e. symmetry such that all directions in any plane perpendicular to x_1 are elastically equivalent. In this case the matrix (1) becomes

$$\begin{array}{cccccc} c_{11} & c_{12} & c_{12} & 0 & 0 & 0 \\ c_{12} & c_{22} & c_{23} & 0 & 0 & 0 \\ c_{12} & c_{23} & c_{22} & 0 & 0 & 0 \\ 0 & 0 & 0 & c_{44} & 0 & 0 \\ 0 & 0 & 0 & 0 & c_{55} & 0 \\ 0 & 0 & 0 & 0 & 0 & c_{55} \end{array}$$

with

$$c_{44} = \frac{1}{2}(c_{22} - c_{23}).$$

This matrix has only five independent, non-zero constants.

III. EXPERIMENTAL

The specimens used were small parallelepipeds of ram's horn, with thickness between 1 and 3 mm and other dimensions between 5 and 20 mm; the small thickness was necessitated by the high attenuation of transverse waves in the horn. Their edges were aligned as accurately as possible along the principal directions r , θ , z of the horn. Specimens 1 to 6 were cut from one large horn of the pair which had been used for the measurement of the dilatational constants; specimens 7 and 8 were cut from a smaller and less homogeneous horn, in which the sheets of cells described in Part I as lying perpendicular to r were not flat, but were bent into a wavy form in the $r\theta$ plane.

The velocities of shear waves propagated in the direction of the thickness of each specimen were measured by the use of apparatus developed and constructed at the National Physical Laboratory, a modified form of the apparatus described by Bradfield (1950). The specimen was lightly oiled* to establish good acoustic contact and gripped between two steel probes along which was transmitted a pulsed, transverse, elastic wave of frequency 4 Mc/s. The specimen was so orientated that the direction of vibration in the probes was parallel to a principal direction of the horn. The time of transit of the pulse through the specimen and the thickness of the specimen while it was gripped by the probes were measured, and hence the velocity of propagation was calculated.

Some difficulties were encountered in the application of the method to horn. The most serious was that the measured velocity was found to be sensitive to the pressure exerted by the probes on the horn, an effect which had not been encountered with the harder solids for which the apparatus was designed. It was found necessary to adjust the pressure by means of springs to an approximately constant value of about 4×10^8 dyn cm⁻².

No correction was applied for end effects due to surface films etc., since these corrections had been found by Bradfield (1950) to be small in this apparatus.

The relative humidity and the temperature were not closely controlled, as they are not important at these frequencies (see Part I). The specimens were stored and used at about 20°C and between 35 and 60 per cent. R.H.

IV. RESULTS

(a) *Velocities*

The values of the shear wave velocities determined at a fixed pressure are shown in Table 1 and Figure 1(a). The random errors in the individual measurements were small (probable error $\leq 0.004 \times 10^5$ cm/sec in all but one case, in which it was 0.014×10^5), but duplicate measurements on different specimens differed by a greater amount, owing apparently to inhomogeneity of the horn.

* The present, improved practice in the National Physical Laboratory is to use a solid transfer film, instead of oil, for weak solids; this permits lower pressures between the probes and the specimens.

These data show that most of the principal transverse wave velocities are close to 1.3×10^5 cm/sec, but one is significantly larger: $\omega_{z\theta}$, the velocity of a wave propagated in the z direction with vibration parallel to θ , is 1.42×10^5 cm/sec (range 1.40 to 1.44).

TABLE 1
VELOCITIES OF TRANSVERSE WAVES, $\times 10^{-5}$ CM SEC $^{-1}$, AT FIXED PRESSURE

Direction of propagation		r	r	θ	θ	z	z
Direction of vibration		z	θ	z	r	r	θ
Specimen	Thickness (cm)						
1*	0.308						
2	0.223			1.33	1.31		
3	0.259	1.29	1.26				
4	0.200	1.32	1.29				
5.	0.258					1.25	1.40
6	0.112					1.25	1.44 ± 0.03
Means		1.30_5	1.27_5	1.33	1.31	1.25	1.42

Error (1 in 20 level) for each individual measurement was $\leq 0.01 \times 10^{-5}$ cm sec $^{-1}$ except where otherwise indicated.

* Specimen 1 was damaged before measurement.

Three other sets of measurements confirm that $\omega_{z\theta}$ is greater than the other velocities; these are: (i) the less accurate measurements on specimens 1 to 6 which are shown in Figure 1(b). These were made without standardizing the pressure or correcting for compression of the horn; (ii) some approximate measurements on specimens 7 and 8, which are also shown in Figure 1(b). These were preliminary measurements made by a different observer, Dr. G. Bradfield, of the National Physical Laboratory, on specimens cut from a different horn, one which had a poorly oriented structure (see last section); (iii) calculation from the observations made during measurement of the dilatational velocities, which gave $\omega_{z\theta}$ about 1.6×10^5 cm/sec as against about 1.3 or 1.4×10^5 cm/sec for the other velocities. In this case there was no static pressure.

In none of these three sets of measurements can much significance be attached to the absolute values of the velocities, but their relative values, which are more accurate, confirm that $\omega_{z\theta}$ is higher than the other velocities.

The data of Figures 1(a) and 1(b) show a small difference between $\omega_{r\theta}$ and ω_{rz} , to which significance must be attached since the compared velocities were measured on the same specimens, so excluding the effect of variation of the horn.

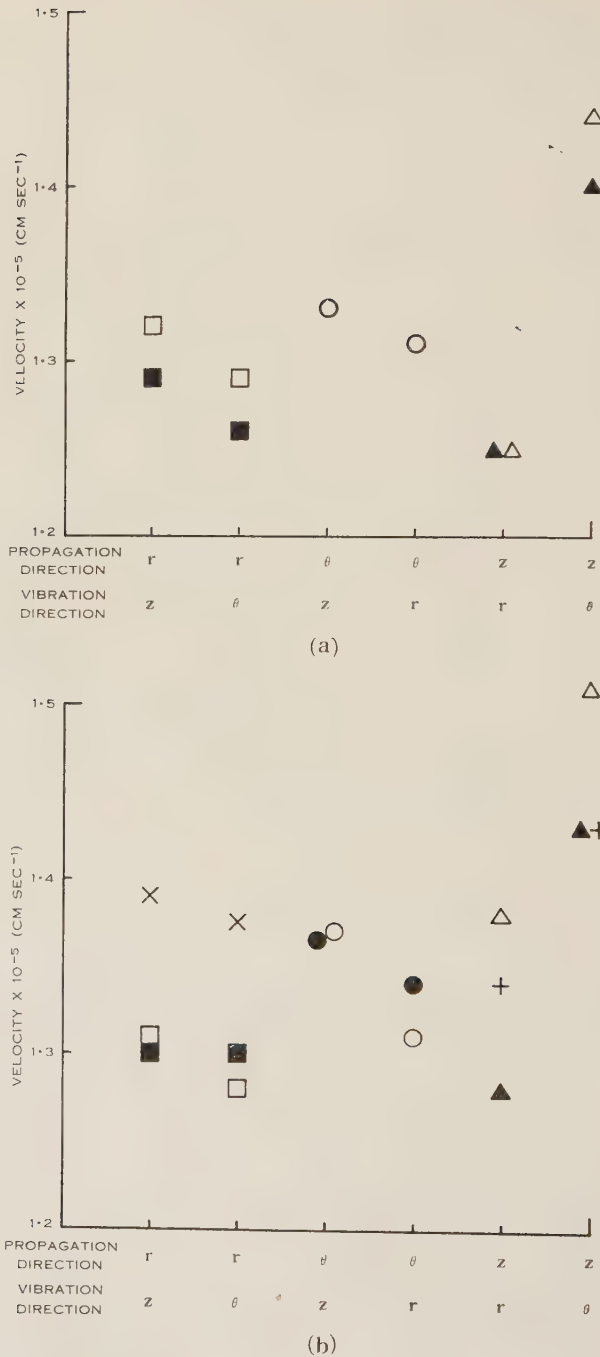


Fig. 1.—The velocities of transverse waves in ram's horn: (a) at fixed pressure of 4×10^8 dyn cm $^{-2}$; (b) less accurate values obtained without standardizing the pressure. Specimens 1 to 6 cut from one horn, specimens 7 and 8 from another, of inferior orientation.

Specimen	1	2	3	4	5	6	7	8
Symbol	●	○	■	□	▲	△	×	+

The small differences shown in Figure 1(*a*) between ω_{zr} and ω_{rz} , and between $\omega_{r\theta}$ and $\omega_{\theta r}$, cannot be considered significant, as the compared measurements were made on different specimens. It was not possible in the time for which the apparatus was available to make a thorough statistical test of these differences.

(*b*) Elastic Constants

In order to calculate the elastic constants it is necessary to know the crystallographic symmetry of the horn. This is discussed in the next section, where it is shown that it is a reasonable approximation to put the shear cross-constants equal to zero, and to calculate the rigidity constants from the equations

$$c_{44} = \rho \left(\frac{\omega_{\theta z} + \omega_{z\theta}}{2} \right)^2; \quad c_{55} = \rho \left(\frac{\omega_{zr} + \omega_{rz}}{2} \right)^2; \quad c_{66} = \rho \left(\frac{\omega_{r\theta} + \omega_{\theta r}}{2} \right)^2,$$

where ρ is the density of the horn, taken as 1.30 ± 0.02 gm cm⁻³ (see Part I). The values so calculated are shown in Table 2. It is clear that c_{55} and c_{66} are equal within the accuracy of the data, and less than c_{44} .

It will be shown in the next section that ram's horn is approximately transversely isotropic about r , so that the constant c_{23} ($= c_{\theta z}$) can be calculated approximately from the relation

$$c_{44} = \frac{1}{2}(c_{22} - c_{23}).$$

TABLE 2

THE PRINCIPAL RIGIDITY CONSTANTS OF RAM'S HORN

Calculated with the assumption of transverse isotropy about radial direction

Constant	Shear Plane	Value of Constant $\times 10^{-10}$ (dyn cm ⁻²)
c_{44}	θz	2.46 ± 0.2
c_{55}	zr	2.13 ± 0.1
c_{66}	$r\theta$	2.16 ± 0.1

The limits given correspond to the range of the observations at fixed pressure.

Values of c_{22} ($= c_{\theta\theta}$) have been determined by both pulse and continuous wave techniques (see Part I); it is appropriate to use the former value, $c_{22} = (1.05 \pm 0.01) \times 10^{11}$ dyn cm⁻², since the conditions under which this value was obtained were similar to those of the transverse wave measurements, although the static pressure was not as high. This value of c_{22} gives $c_{23} = (5.6_5 \pm 0.1) \times 10^{10}$ dyn cm⁻². In view of the approximations of the argument and the sensitivity of c_{23} to the value adopted for c_{22} , which itself depends on pressure, this value of c_{23} should be treated with some reserve; e.g. adoption of the continuous wave value $c_{22} = 1.01 \times 10^{11}$ dyn cm⁻², determined under zero static pressure, would have given $c_{23} = 5.2 \times 10^{10}$ dyn cm⁻². It seems probable that c_{23} under a static pressure of about 4×10^8 dyn cm⁻² lies in the range $(5.7 \pm 0.5) \times 10^{10}$ dyn cm⁻².

V. THE SYMMETRY OF RAM'S HORN

The values of the dilatational elastic constants reported in Part I indicated that ram's horn is transversely isotropic about the radial (r) direction. If this were strictly true, the "transverse" waves in the principal directions would be strictly transverse, and their velocities would satisfy the relations

$$\omega_{rz} = \omega_{zr} = \omega_{r\theta} = \omega_{\theta r} ; \omega_{\theta z} = \omega_{z\theta},$$

where ω_{ij} is the velocity of a wave propagated along the x_i -axis with vibration direction parallel to x_j .

The results quoted in the last section show that $\omega_{\theta z} \neq \omega_{z\theta}$ and $\omega_{rz} \neq \omega_{r\theta}$. It is highly improbable that these inequalities are due merely to misalignment of the specimens, since they have been found for different specimens and, in the former case, by different methods of measurement. The most probable explanation is that the symmetry of ram's horn is rather low, being that of a crystal system for which the shear cross-constants are not all zero.

In order to calculate the rigidity constants from the measured velocities, the approximation has been made of putting the shear cross-constants equal to zero, which would entail $\omega_{ij} = \omega_{ji}$, and using the mean of ω_{ij} and ω_{ji} in the calculations. Since the differences between ω_{ij} and ω_{ji} are not large, the error introduced by this approximation should be small, as may be seen by comparison with a rather similar problem considered by Fein and Smith (1950). There would be little point in attempting to improve the estimates of the rigidity constants at present, as the horn itself is so variable.

The data of Table 2 show that within the accuracy of this approximation ram's horn is transversely isotropic about r , as was concluded from measurement of the dilatational constants.

VI. CORRELATION OF THE ELASTIC ANISOTROPY WITH THE STRUCTURE

It was shown in Part I that if the elastic anisotropy of ram's horn was primarily determined by its molecular structure, its symmetry would approximate to transverse isotropy about the growth direction z , whereas if it were determined by the histological structure the symmetry would approximate to transverse isotropy about the radial direction r ; the latter was observed. This observation is confirmed by the data on the shear wave velocities presented here.

The small departure from strict transverse isotropy about r does not contradict the conclusion that the anisotropy is determined primarily by the histological structure; it is to be expected that the spiral manner of growth of ram's horn will introduce some small departure of the crystallographic axes from orthogonality.

The correlation of the anisotropy with the histological structure can be carried a little further. The structure of ram's horn, described in Part I, approximates to a pile of sheets of keratin lying perpendicular to r , comparatively weakly joined together along r . Since the molecular chains lie in the plane of the sheets, the constant c^h_{rr} will be less than $c^h_{\theta\theta}$ or c^h_{zz} , where the superscript h refers to the material of the sheets. All three constants for the pile of sheets will be reduced by the lamination of the structure, but it can easily be shown

that c_{rr} will be reduced more than c_{HH} or c_{zz} . Hence, for the horn as a whole, c_{rr} should be less than $c_{\theta\theta}$ and c_{zz} , as is in fact observed.

In the case of the rigidity constants it is not possible to say with certainty which of the constants c_{44}^h , c_{55}^h , and c_{66}^h would be greatest, but it is probable that c_{44}^h would be, since the crossed or dispersed molecular chains lie in planes perpendicular to r . It can be shown that c_{55} and c_{66} will be reduced by the lamination of the structure more than c_{44} , so it is very probable that for the horn as a whole c_{44} would be greater than c_{55} and c_{66} , as is in fact observed.

VII. COMPARISON WITH RESULTS OBTAINED BY OTHER WORKERS

Very few measurements have been made of the rigidity constants of keratin. All previous ones have been made on wool or hair fibres at frequencies of the order of 0.1 c/s, and only the torsional rigidity about the fibre axis (c_{44}) has been determined. The weight of the evidence (Auerbach 1923 (after correction); Peirce 1923; Herzog 1928; Speakman 1929; Ray 1947; Lochner 1949; Meredith 1954) is that under these conditions and at 20°-25°C, c_{44} for keratin fibres is about 1.8×10^{10} dyn cm⁻² in the completely dry state and 1.0×10^{10} dyn cm⁻² at a relative humidity of 65 per cent.

The structure of keratin fibres is different from that of ram's horn, and they would be expected to be transversely isotropic about the fibre axis, so that this modulus is not exactly comparable with any of the values $c_{44} = 2.4_6$, $c_{55} = c_{66} = 2.1_5 \times 10^{10}$ dyn cm⁻² found here for ram's horn. However, they should not differ much under the same conditions, as the anisotropy is not marked in either case. The large difference observed is to be ascribed to the very large difference in the frequency of the measurements, relaxation processes which occur in the keratin at low frequency being unable to follow the high frequency. Such dispersion of the elastic constants is common in high polymers, and is also shown by the dilatational constants of keratin (see Part I). These relaxation processes in keratin can also be blocked to some extent by a decrease of regain, as is shown by the steep increase in the low-frequency elastic constants with decrease in regain. Under completely dry conditions, c_{44} for the fibres at 0.1 c/s (1.8×10^{10} dyn cm⁻²) is approaching the value of c_{55} or c_{66} (2.1×10^{10} dyn cm⁻²) determined for ram's horn at 4 Mc/s.

It is not possible to make any comparison of the constant c_{23} determined here with the value of the Poisson ratio $\sigma_{z\theta}$ which was measured by Warburton (1948) for horn, since $\sigma_{z\theta}$ cannot be calculated from c_{23} until the remaining constant c_{12} has been measured independently.

VIII. CONCLUSIONS

The measurements of the dilatational elastic constants of various forms of keratin at 4 and 5 Mc/s, which were reported in Part I, led to the conclusion that the elastic anisotropy of keratin in the region of small strains depended at least as much on its histological as on its molecular structure. The measurements of the rigidity constants of ram's horn reported here support this conclusion. The elastic behaviour again approximates to transverse isotropy about

the radial direction, which is histologically unique, rather than about the growth direction, which is molecularly unique. There is a small departure from strict transverse isotropy, which is to be expected in view of the spiral structure of ram's horn. The relative magnitudes of both the three dilatational and the three rigidity constants are such as would be expected in view of the histological structure of ram's horn.

The rigidity constants of ram's horn are: $c_{44} = (2.46 \pm 0.2) \times 10^{10}$; $c_{55} = (2.13 \pm 0.1) \times 10^{10}$; $c_{66} = (2.16 \pm 0.1) \times 10^{10}$ dyn cm⁻²; these constants refer to shear in the θz , zr , and $r\theta$ planes respectively, and have been determined at a frequency of 4 Mc/s, a temperature of about 20°C, and relative humidities between 35 and 60 per cent., under a static pressure of 4×10^8 dyn cm⁻². They have been calculated by making the approximation that the shear cross-constants are zero. The limits quoted correspond to the range of the observations.

These values are higher by a factor of two than the values, determined by other workers, of the torsional rigidities of wool and other keratin fibres about the fibre axis at frequencies of the order of 10^{-1} c/s and at about 65 per cent. R.H.; this is ascribed to the blocking of relaxation processes at the higher frequency.

The elastic constant c_{23} , which measures the tensile stress in the x_2 or θ direction per unit extension in the x_3 or z direction, has been roughly evaluated by making the approximation that ram's horn is transversely isotropic about x_1 or r . It probably lies in the range of 5.2 to 6.2×10^{10} dyn cm⁻².

No estimate can be made from these data of the remaining constant c_{12} .

IX. ACKNOWLEDGMENTS

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THE BILATERAL STRUCTURE OF WOOL CORTEX AND ITS RELATION TO CRIMP

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Summary

New evidence of the nature of the bilateral cortex in crimped wool is presented and discussed in relation to the physical and chemical properties of the wool fibre. The origin of the asymmetry in the cortex was studied by cutting cross sections of sheep skin and swelling plucked wool roots. It is shown that the bilateral structure is present before the cortex is keratinized and does not originate from an asymmetrical keratinization as has been suggested. Coarse wools, in which crimp is much less, are shown to possess a radial asymmetry in which the peripheral cortical cells are less accessible to basic dyes and have a higher sulphur content than the central cells. This is related to the difference between the curved follicles from which fine fibres are produced and the straighter follicles producing coarse fibres.

I. INTRODUCTION

It has been demonstrated that the cortex of a crimped wool fibre comprises two hemi-cylinders differing in both chemical and physical properties, this structural feature bearing a direct relationship to the occurrence of crimp (Horio and Kondo 1953; Mercer 1953*b*, 1954; Fraser and Rogers 1953, 1954). These differences between the two cortical components can be revealed *inter alia* by the segmental dyeing produced under controlled conditions, or by numerous keratin swelling or digesting agents applied to intact fibres and fibre cross sections. Horio and Kondo (1953), following the earlier studies of Hirabayashi (1938) and Ohara (1938), confirmed that in wool fibre cross sections one segment was more accessible to basic dyes than the other and this was termed the dye-accessible segment or D.A. This segment was always situated on the outside of the crimp wave and they claimed that the dyeing pattern was reversed with acid dyes.

The present authors prefer the terms H ("hard") and S ("soft"), the S segment being that segment of the cortex which lies on the outside of the crimp wave, and this terminology will be used throughout this paper. H and S refer to properties of the cortex and reflect the differential properties of the segments as related to wool fibre performance, that is, chemical resistance and mechanical strength. It is not intended, *a priori*, to relate these terms to the established terms of hard and soft keratins used in histological descriptions of the two main keratin types derived from different epidermal tissues. Neverthe-

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less, the differences in properties of the two cortical segments can in part be related to a higher degree of disulphide cross-linking or keratinization.

Experimental results up to the present time suggest that there are two major contributory causes to the appearance of segmentation. One is a fundamental chemical difference in the amino acid composition, particularly in cystine, proline, and dicarboxylic amino acids (Fraser, Lindley, and Rogers 1954) and the other a histological feature, associated with chemically resistant membranes surrounding the cortical cells (Manogue and Moss 1953) and resistant particles in the cells (Mercer 1953*a*). The resistant particles have been called non-keratinous and have been claimed to exist in greater abundance in the more stable cortical segment (Mercer 1953*b*). The resistant cortical cell membranes which are of the same order of thickness as the epicuticle have been observed in cross section in the electron microscope (Manogue and Moss 1953) and although there is no evidence for any segmental disposition of these membranes, differences in dye accessibility and resistance to swelling agents may be related to their presence. It is the purpose of the present paper to coordinate and extend published information on this subject and to present a photomicrographic record of some of the most important features of wool-fibre histology interpreted in terms of segmentation and the occurrence of crimp.

II. EXPERIMENTAL

Selected samples of various quality wools and human hair were solvent scoured with 80-100°C b.p. petroleum ether at room temperature, washed several times with distilled water, dehydrated with alcohol, and air dried. Differentiation of the segments was obtained by dyeing with 0.1 per cent. methylene blue in 0.03M phosphate buffer, pH 7.4, at 100°C for 30 min with a wool/liquor ratio of 100 mg/30 ml. A qualitative indication of disulphide bond distribution was obtained by oxidizing with 1.6 per cent. peracetic acid at room temperature for 24 hr with a wool/liquor ratio of 1 g/100 ml and dyeing with 0.0005M methylene blue or toluidine blue at room temperature for 30 min with slight agitation. Cross sections cut in a Hardy microtome were then investigated for dye distribution.

Experiments on cortical segmentation in the follicle were conducted on 8 μ paraffin sections cut from freshly collected sheepskin, showing Merino type crimp, which had been fixed in 5 per cent. formol-saline for 24 hr and treated in the usual way. For staining, the sections were taken down to water and oxidized in 1.6 per cent. peracetic acid for 24 hr. and then thoroughly washed in water. They were then stained in 0.0005M methylene blue (0.1M acetate buffer at pH 2.6) for 2 hr, followed by Mayer's haemalum and finally eosin as counterstain. Freshly collected wool roots were also treated by several methods before examination.

III. RESULTS AND DISCUSSION

(a) Staining Reactions

(i) *Basic dyes*.—In Merino 64's quality wool there is a marked difference between the basic dye affinity of the H and S segments as shown in Plate 1,

Figure 1, and under the dyeing conditions specified, uptake of basic dye is practically confined to the S segment and the cuticle. In the S segment there is some differentiation of histological detail as the intercellular cementing material and the contents of the nuclear spaces have dyed more intensely than the cortical cells themselves. In the H segment there is also some dye uptake by the intercellular cementing material and the contents of the nuclear spaces.

The marked difference in basophilia between the segments appears to be localized in the cortical cells, and the boundary between the segments follows the outline of cortical cells. A sharp division of basophilia is apparent and no gradation of properties between the two types of cells has been observed in fibres below about $20\text{--}25\ \mu$ in diameter. The cross sectional area of the H segment is, in general, somewhat smaller than that of the S segment and the boundary between the segments is parallel to the major axis of the somewhat elliptical cross section. The disposition of the S segment towards the outside of the crimp wave is shown in Plate 1, Figure 2.

(ii) *Basic Dyes following Oxidation.*—The marked change in the dyeing pattern following oxidation of wool with peracetic acid is of considerable interest. Unlike the staining without oxidation, frequent washing with water did not remove the dye and demonstrated the increased affinity conferred by the oxidation process. Intense staining occurred first in the cuticle and tended to be metachromatic, and maximal staining took place in this structure before cortical staining began. The metachromatic staining was especially obvious with toluidine blue, and was very marked if the oxidized fibres were first swollen with N NH_4OH in order to remove 90 per cent. of the fibre protein and to isolate the cuticular sheaths and cortical cell remnants, and then stained (Plate 1, Fig. 3).

The final staining result with oxidized fibres is shown in Plate 1, Figure 4. It is seen that an intense basophilia has arisen in one cortical segment as well as in the cuticle and that this is more intense than before oxidation. The segment now stained is the H segment (and again is shown to be smaller than the S segment). The identity of the segment has been conclusively demonstrated by carrying out the oxidation with peracetic acid on cross sections of methylene blue dyed fibres whilst observing under the microscope. In this way actual transfer of dye could be observed as it was released from the S segment by the acid reagent and was taken up by the now oxidized H segment.

It is of interest to note that no difference in the segmental staining pattern is observed with the methylene blue solution buffered at pH 7 or at pH 2.6 as employed by Pearse (1951) in his method for staining keratin and adapted by Lillie, Bangle, and Fisher (1954) for the same purpose. These authors did not observe the segmental staining as they used hairs in their investigations.

Mercer (1953*b*) suggested that segmentation is revealed in cross sections of peracetic acid oxidized fibres if these are swollen in 0.1N NH_4OH , but this is difficult to see in the photomicrographs published by this author and he did not identify the segments. If, however, the peracetic acid treated fibres are first stained as described above, the marked preferential swelling of the unstained S segment produced by 0.1N NH_4OH can be readily followed and

always produces bursting of the cuticle on the S side of the fibre (Plate 3, Fig. 1). This also explains why the action of dilute ammonia on peracetic acid oxidized fibres often leads to longitudinal splitting of the fibre cuticle and liberation of the cuticle as a flat unfolded sheath (Plate 1, Fig. 3).

The reason for the observed increase in basophilia after peracetic acid oxidation is still unknown. Alexander, Fox, and Hudson (1950, 1951) did not find free sulphonic acid groups in completely oxidized wool as evidenced by the inability of this wool to undergo ion-exchange reactions characteristic of a product containing free sulphonic acid groups; however, free cysteic acid was found on acid hydrolysis. Lillie *et al.* (1954) in their study of this increased basophilia in various keratin structures concluded that sulphonic acid groups were the only ones which could account for the reactions observed, particularly since basic dye staining occurred at low pH values, as observed in the present work.

We found that wool which had been fully reduced with thioglycollic acid and coupled with iodoacetamide did not show any increase in basophilia after treatment with peracetic acid. It seems therefore that at least some free cysteic acid side-chains must be formed in oxidized keratin, and the distribution of the resultant basophilia gives a qualitative indication of the degree of disulphide cross-linking. If this is so, then the results with fine Merino wool indicate that the disulphide cross-linking is greater in the H than the S segment. This observation is further supported by a marked increase in the basophilia of the H segment after the reduction of the disulphide bonds with thioglycollic acid at pH 5.6 followed by coupling with iodoacetic acid (O'Donnell 1954). The increase in the basophilia of the H segment was demonstrated by dyeing with basic dyes at pH 7, but no dyeing occurred at pH 2.6 as with peracetic acid oxidized wool. This is in accord with the difference in the pK values of the sulphonic acid and carboxylic acid groups introduced by these procedures. The distribution of disulphide linkages in the wool fibre cortex revealed by these staining methods has been correlated with sulphur analyses carried out on two fractions obtained from wool by the action of cetyl sulphonic acid in which the segments are separated on the basis of alkali solubility (Fraser, Lindley, and Rogers 1954). The differing physical and chemical properties of the bilaterally constructed cortex are without doubt related in part to this difference in the degree of keratinization.

(iii) *Fibre Types and Cortical Segmentation.*—Plate 3, Figures 2 and 3, shows the uptake of basic dye in Corriedale 56's quality wool before and after oxidation and Plate 3, Figures 4 and 5, shows that in Lincoln wool. It appears from the study of a large number of cross sections that the disappearance of segmentation, as seen in Merino 64's quality wool, is a function of fibre diameter rather than breed and Plate 3, Figure 3, shows this feature particularly well. In fibres less than about 25μ in diameter the segments are well formed and similar to those of Merino 64's quality.

In the diameter range $25\text{--}35\mu$ the sharp demarcation of the segments gradually disappears and the uptake of dye in individual cortical cells is somewhat variable although a classification into H-type cells and S-type cells is

still possible after oxidation. Frequent examples of an H cell surrounded by S cells and an S cell surrounded by H cells are seen in the coarser fibres in Plate 3, Figures 3 and 5. The population of H cells is somewhat less than that of S cells as in the Merino 64's quality fibres.

Above a diameter of about 35μ a division into H and S segments can no longer be justified on the basis of the staining methods described. However, H and S cells may still be differentiated both on the basis of basophilia and disulphide bond content. In the coarser Lincoln fibres of Plate 3, Figures 4 and 5, there is a marked tendency for the H cells to be concentrated around the periphery of the cortex leaving a central core of S cells (cf. Mercer 1952).

The examples of asymmetrical dye uptake discussed above were chosen from a wide range of fibre types as they illustrate the general trends in passing from fine highly crimped wools to coarser wools in which the crimp is much less developed. An additional observation worth recording concerns the "steely" wool produced by sheep maintained on a copper deficient diet, the crimp in this type of wool being very much less than is usual for the particular breed. In the two cases studied ample copper had been restored to the diet of the sheep at a later stage. The later growth was highly crimped, serving as a control. The effect of copper deficiency varied considerably from fibre to fibre; in some cases the segments were ill formed as in coarse wools and in others the difference in basophilia between H cells and S cells was only slight. Similarly anomalous results were obtained after oxidation (Plate 1, Fig. 5).

(b) The Action of Alkalis and Urea

The swelling and breakdown of wool fibres in alkaline media are quite different from that observed in acid media. In general, cortical cells are not released on hydrolysis and the bulk of the protein released appears to be intracellular in origin. A resistant intercellular residue surrounded by a cuticle sheath remnant is commonly observed after alkaline treatments, as for example in Plate 1, Figure 6.

The H and S segments of the cortex differ markedly in their resistance to swelling in dilute alkali. Immersion in 0.1N NaOH for 1-2 hr produces a gross swelling of the S segment with greatly reduced birefringence, whilst the H segment is comparatively unaffected by this treatment. In the presence of thioglycollate the effect is accelerated and is accompanied by the development of blisters on the cuticle around the S segment (Plate 3, Fig. 6).

An electrophoretically pure component extracted from Merino 64's wool with alkaline thioglycollate solution (Gillespie and Lennox 1953) has been shown to contain more aspartic and glutamic acids and amide N than the intact wool, but less cystine and proline (Simmonds 1955). Since this is believed to be the major protein component of the wool fibre and its removal coincides with a depletion of the S segment, these differences in amino acid composition between component 2 (Gillespie and Lennox 1955) and the whole fibre must reflect similar differences between the H and S segments.

Differentiation similar to that obtained with 0.1N NaOH is obtained by treatment with saturated aqueous urea solutions at 65°C for several days. The addition of a secondary alcohol sulphate-type detergent to the urea solution

accelerates the differentiation and finally the S segment is swollen to about eight times the original volume, whilst the H segment is not visibly affected apart from the development of striations. At this stage the wool fibres may readily be disintegrated by mechanical agitation with glass beads. The disintegrated products include cortical cells derived from the H segment and fragments of cuticle adhering to a jelly like mass which is presumably derived from the swollen S segment.

(c) The Action of Enzymes and Microorganisms

Wool fibres are slowly digested by crude trypsin solutions and certain types of bacteria and fungi with the release of scales and cortical cells. By means of the various methods of differentiating the H and S segments already described it is possible to show that in partly digested wool fibres the S segment is more susceptible to attack and that the first release of cortical cells is from this segment. This is found to be true in crude trypsin digests obtained under sterile conditions, as well as in the case of bacterial and fungal (McQuade, unpublished data) attack on the fibre.

(d) Mechanical Properties

No detailed investigations of the mechanical properties of the H and S segments have so far been attempted, but a number of preliminary observations suggest that they differ to some extent, which is not surprising in view of the gross chemical and histological differences that have been observed. Dr. M. E. Hargreaves, Division of Tribophysics, C.S.I.R.O., attempted the difficult task of performing microhardness tests on thin sections of fine Merino wool. Satisfactory indentations were obtained on a small number of fibres and two depths of indentation were in general obtained on any one section. The ratio of hardness was of the order of 2 : 1. This is attributed to the greater hardness of the H segment. It is hoped to continue this investigation when a more suitable embedding medium has been developed.

A considerable difference in mechanical properties is observed when Merino fibres are extended 50 per cent. in neutral 0.1M thioglycollate solutions. The H segment is highly birefringent whilst the birefringence of the S segment is reduced and accompanied by the development of striations (Plate 4, Fig. 1).

(e) The Action of Acids

When wool fibres are exposed to solutions of low pH, swelling and progressive degradation of the keratin occurs. Under these conditions the H and S segments can be differentiated by differential swelling, an unsymmetrical development of striations, and differential loss of birefringence, indicating a differing susceptibility to acid hydrolysis. Prolonged treatment with dilute mineral acids, or shorter treatment with hydrolytic agents having a catalytic action, result in the release of cortical cells and scales. Again a difference in resistance is observed, indicating that the material of the segments differs considerably.

The differing swelling properties of the bilateral cortex in acid solutions are readily demonstrated by immersing fibres in glacial acetic acid, when a rapid swelling of the S segment is observed, accompanied by a loss of birefringence.

Prolonged treatment with 0.1N HCl or 0.1N oxalic acid at 65°C results in the release of cortical cells. With cetyl sulphonic acid a rapid action is obtained and the two segments are revealed (Plate 2, Fig. 1). Two fractions have been isolated from the wool after treatment with 0.05M cetyl sulphonic acid for 6 days at 65°C (Lindley 1947). One fraction is soluble in dilute alkali and has been shown by microscopical examination after basic dye staining to be derived mainly from the S segment, whilst the insoluble fraction is derived mainly from the H segment and cuticle (Fraser, Lindley, and Rogers 1954).

Amino acid analyses of these fractions have revealed gross difference in the composition of the segments. Thus the resistant fraction is rich in cystine and proline, whereas the alkali-soluble material is lower in cystine but has a higher content of glutamic and aspartic acids. The analytical results are in accord with the segmental staining results discussed above (Section III, (a)), which indicate a high sulphur content in the H segment but a greater basic dye affinity in the S segment. The results can also be correlated with the generally accepted view that the stability of wool keratin towards chemical reagents is largely due to the numerous disulphide linkages which exist both inter- and intramolecularly.

(f) *Further Experiments to Reveal Segmentation in Wool Cortex*

Four further methods aimed at detecting chemical differences in the two segments as well as differences in accessibility to chemical reagents depending on histological features were unsuccessful. Wool stained with (i) ninhydrin by the method of Cockburn, Jagger, and Speakman (1953), (ii) iodine by the method of Richards and Speakman (1953), and (iii) 2:4 dinitrofluorobenzene by the method of Middlebrook (1951), and studied in cross sections failed to show segmentation. In the last case, reduction of the DNP-wool with stannous chloride followed by coupling with H-acid produced no change in the staining result and so no localized distribution of side-chain $-NH_2$ groups could be established. Wool stained by immersion for 7 days in 1 per cent. osmic acid buffered in 0.1N veronal at pH 7.4 again showed only a generalized staining of the cortex in cross section.

The action of saturated chlorine or bromine water on wool fibres readily produces segmentation by preferential attack on one of the segments, the action of bromine being the most rapid and destructive to the keratin. It is of interest to note that initially the blister formation (Allwörden reaction) induced by this halogen occurs only on the S side and later spreads all around the fibre surface (Fraser and Rogers 1955). Although this was suggested to indicate differences in the cuticle around the H and S segments (Leveau, Cebe, and Parisot 1953), the most likely explanation is a difference in the molecular size and concentration of the osmotically active substances liberated in the H and the S segments (Fraser and Rogers 1955). This is probably due to differences in the amino acid composition of the two segments.

(g) *The Origin of Segmentation*

Wool follicles producing crimped wool fibres have a characteristic histological structure which must play a role in the mechanism responsible for crimp

formation. This includes the bulb deflection and bending of the follicle, and the process of asymmetric keratinization of the fibre cortex (Auber 1951). This invariably begins on the thin side of the asymmetric inner root sheath and progresses to the thick side. The cross-sectional dimensions of the outer root sheath on the other hand are variable and take no part in the mechanism. Now that it is known that the wool fibre is bilaterally constructed, it is certain that these factors are responsible for the "setting" of the two segments in a fixed relationship to one another, once they have been formed. Experiments on the swelling and birefringence changes of plucked wool roots induced by various agencies (Plate 2, Fig. 2; Plate 4, Fig. 2) have led to the belief (Fraser and Rogers 1954) that the chemical differences in the proteins of the two segments originate at very early stages of the biosynthesis of the cortex where fibrillation begins and the sulphydryl reaction can be first detected (Plate 2, Fig. 3). They do not arise as a result of the final hardening process of keratinization which occurs in the lower third of the follicle (Mercer 1954). Furthermore, it seems that keratinization of the S segment may not follow immediately upon its completion in the H segment, these processes occurring in distinct steps rather than continuously across the developing fibre (Plate 2, Fig. 2).

Naturally, it can be suggested that if the bulb deflection is important in the crimping mechanism, then, within one region of the skin where stable crimp exists and fibre crimps are in phase, the follicle bulbs should all be deflected in the same direction. However, this is not found and is not necessarily a prerequisite. Not only are the follicle bulbs at different levels enabling "out of phase" growth, but fibre growth rates are not the same due to follicle-follicle competition, and in any case the staple crimp results from fibre interaction and is not a time indication of "phasing" of the crimp waves from adjacent follicles.

It is now generally accepted that the most chemically resistant segment of the wool cortex (H) is formed on the concave side of the bent follicle, adjacent to the thin side of the inner root sheath. Until recently the evidence for this has been only circumstantial and it has been assumed that the first segment to keratinize should be the H segment as a consequence of its greater degree of disulphide cross-linking. Conclusive evidence that this is the true situation has been presented by Fraser and Rogers (1954) who revealed segmentation in fine Merino wool fibres in cross sections of the wool follicle by the peracetic acid oxidation-basic dye method. It was demonstrated that, almost without exception, the increased basophilia of the H segment was on the thin side of the inner root sheath, and the boundary between the two segments was again situated along the major axis of the elliptical fibre cross section. The presence of the H segment and its disposition in relation to the follicle layers was observed at all levels of the follicle except at the very early stages of fibre formation.

If it is accepted that the action of oxidizing per-acids on wool keratin is to produce side-chain sulphonc groups or at least that the increase in basophilia detected by the basic dye staining at pH 2.6 is specific for acidic groups derived from sulphur-containing side-chains, then such a method should be capable of detecting the level in the wool root at which incipient keratinization takes place. It has been found that when this technique is applied to

sheepskin sections cut at the root level, regions of basophilia, indicating a disulphide content, arise in the presumptive cortex before the morphological changes of keratinization have advanced very far. (Plate 2, Figs. 4 and 5.) Moreover, these regions again appear always on the thin side of the inner root sheath (presumptive H segment) finally spreading until the basophilia completely fills the whole H segment when the fibre has finally keratinized (Plate 2, Fig. 6).

(h) The Origin of Asymmetry in the Cortex of Coarse Wools

With sections of sheepskin from a strong-wool Merino it was found that fibres of large diameter (primary follicles) were often stained in a similar way to that already described for coarse wools. However, the more intense staining tended always to be on the thin side of the inner root sheath as is always the case with the secondary fibres of smaller diameter. In straight-haired animals such as the guinea pig no segmental dyeing was observed, the fibre cortex and cuticle being completely stained and centrally placed in the symmetrical inner root sheath. This is similar to the results obtained by Pearse (1951) in his study of the technique as a "keratin" stain and the later investigations of Lillie *et al.* (1954).

The differentiation of the H and S segments in Merino fibres is mainly due to the differing dye affinities of the two types of cortical cells rather than areas of the cortex. It is reasonable therefore to discuss the asymmetry of the cortex in coarse fibres in terms of the two types of cortical cell, bearing in mind, however, that histological entities such as cortical cell membranes and intercellular cementing material may also contribute to the dye accessibility of the cortical cells.

H and S cells may still be differentiated in coarser wools but there is a transition from a bilateral structure to one of radial asymmetry as the diameter increases from about 25-40 μ . This transition may be correlated with the change in follicle structure in passing from those producing highly crimped fibres to those producing straighter fibres in which curvature, bulb deflection, asymmetric keratinization, and eccentric disposition of the developing fibre in the inner root sheath are not so marked (Auber 1951).

As mentioned earlier, we have shown that segmental properties exist at the earliest stages of the differentiation of the cortex (Fraser and Rogers 1954). This, together with the clearly defined boundary between the segments suggests that the cell *type* is predetermined in the germinal layer and does not result from any intra- or extrafollicular influence during development. The *course* of the metabolism and differentiation of each type of cell, however, is almost certainly related to the asymmetry in the follicle.

In coarser wools the division into two cell types is not so well marked in cross section and the formation of a core of S cells is not readily explained. The cell type may be predetermined in the germinal layer of the follicle, as we have supposed in the case of fine wools, or alternatively the central core of S cells could result from a lesser degree of keratinization towards the centre of the fibre during development. The fact that the cells may still be divided into two types, and the occurrence of isolated H and S cells, however, lends

considerable support to the view that the cell type is predetermined in the germinal layer, and that the amount of crimp is a function of the pattern of their distribution in the developing fibre.

(i) *The Isolation of the H Segment*

Mercer (1953*b*) has described a method whereby the S segment of crimped wool, supercontracted in superheated water, can be digested with trypsin, leaving the H segment and cuticle comparatively intact. Analysis of the residue showed that the H segment was considerably richer in cystine than the S segment (Mercer, Golden, and Jeffries 1954), thus confirming the results obtained by Fraser, Lindley, and Rogers (1954). An alternative method (Fraser, Rogers, and Thompson, unpublished data) is to digest the unsymmetrically supercontracted wool with crystalline papain in the presence of urea and bisulphite. The digestion is followed microscopically and when the S segment shows some degradation, the H segment can be isolated by shaking with glass beads and passing the product through a 200 mesh sieve. The isolated H segment, in this case, is not contaminated with cuticle as with Mercer's preparation.

The cystine analyses given by Mercer *et al.* (1954) are open to the criticism that considerable lanthionine formation occurs during the unsymmetrical supercontraction as evidenced by low solubility in alkaline thioglycollate (Lennox, unpublished data) and paper chromatograms (Thompson, unpublished data). The method described by Fraser, Lindley, and Rogers (1954) is also open to criticism and whilst there is abundant supporting evidence for a preponderance of cystine in the H segment, quantitative analysis of the cystine contents of the H and S segments must await better methods of isolation.

IV. ACKNOWLEDGMENT

The authors are indebted to Dr. M. E. Hargreaves, Division of Tribophysics, C.S.I.R.O., for attempting the difficult task of performing microhardness tests on thin sections of fine Merino wool.

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EXPLANATION OF PLATES 1-4

PLATE 1

- Fig. 1.—Cross section of Merino wool fibres stained with methylene blue, showing segmentation and some delineation of cortical cells and nuclear remnants. $\times 240$.
 Fig. 2.—Merino wool fibre stained with methylene blue showing S segment on outside of crimp wave. $\times 240$.
 Fig. 3.—Cortical cell residues and cuticular sheaths from Merino wool oxidized with peracetic acid, extracted with N NH_4OH and stained with methylene blue. $\times 240$.
 Fig. 4.—Merino wool fibres oxidized with peracetic acid and stained with methylene blue. Compared with Plate 1, Figure 1, the H segment is now strongly basophilic. $\times 240$.
 Fig. 5.—Irregular staining and segmentation in "steely" Merino wool treated as in Plate 1, Figure 4. $\times 240$.
 Fig. 6.—Wool fibre residue following extraction with potassium thioglycollate, pH 12.2, as described by Gillespie and Lennox (1953). Stained with methylene blue. Compare with Plate 1, Figure 3. $\times 240$.

PLATE 2

- Fig. 1.—Cross section of cetyl sulphonic acid treated Merino wool fibres stained with toluidine blue. $\times 240$.
 Fig. 2.—Freshly plucked wool root swollen in detergent, and photographed in polarized light with gypsum-red plate. The two prekeratinized segments can be seen as distinct entities with birefringence beginning at different levels. $\times 50$.
 Fig. 3.—Bulb of plucked wool root stained for sulphhydryl groups by the tetrazolium method (Rogers 1953), showing increase in sulphhydryl reactivity with development of fibrillation in the cortex. $\times 240$.
 Fig. 4.—Section of Merino skin in the follicle bulb region stained by the peracetic acid oxidation-basic dye method. This is at a stage where fibrillation begins but before incipient keratinization and any appearance of asymmetry in the inner root sheath. $\times 240$.
 Fig. 5.—Section of Merino skin in the region of incipient keratinization stained as in Plate 2, Figure 4, but showing greatly increased basophilia in one region of the cortex (presumptive H segment) adjacent to the thin side of the inner root sheath. The inner root sheath is not completely keratinized. Unstained regions delineate the cortical cells and stained nuclei are visible. $\times 540$.
 Fig. 6.—Section of Merino skin stained as in Plate 2, Figures 4 and 5, and showing two fibres in a late stage of keratinization. Delineation of the two segments is evident, the H segment being the most basophilic and situated on the thin side of the asymmetric inner root sheaths which are almost completely keratinized. $\times 240$.

PLATE 3

- Fig. 1.—Cross section of wool fibres oxidized with peracetic acid, stained with methylene blue, and swollen in 0.1N NH_4OH . The unstained S segment has swollen and the cuticle has burst open. The resistant H segment remains intact and cortical cell remnants are seen in the S segment. $\times 1175$.
- Fig. 2.—Cross section of Corriedale 56's quality wool fibres dyed with methylene blue. $\times 600$.
- Fig. 3.—Cross section of Corriedale 56's quality wool fibres oxidized with peracetic acid and dyed with methylene blue. $\times 600$.
- Fig. 4.—Cross section of Lincoln wool fibres dyed with methylene blue. $\times 600$.
- Fig. 5.—Cross section of Lincoln wool fibres oxidized with peracetic acid and dyed with methylene blue. $\times 600$.
- Fig. 6.—Appearance of Merino wool fibre during extraction with 0.05M potassium thioglycollate at pH 12.2. The birefringent H segment is on the inside of the curve and the S segment is highly swollen to give an Allwörden reaction-like appearance. $\times 450$.

PLATE 4

- Fig. 1.—Merino wool fibre stretched to 50 per cent. extension in 0.1M potassium thioglycollate at pH 7. The two segments can be seen with striations in the S segment. $\times 3000$.
- Fig. 2.—Freshly plucked wool root swollen in detergent. The presumptive S segment has dispersed leaving the more stable presumptive H segment. $\times 250$.

BILATERAL STRUCTURE OF WOOL CORTEX

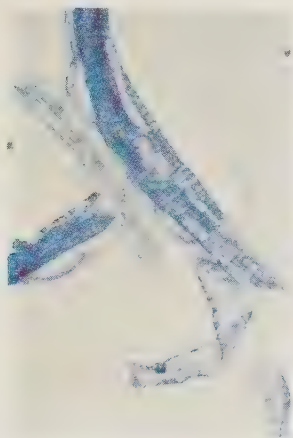


Fig. 3

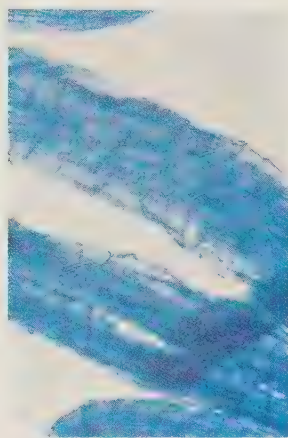


Fig. 6

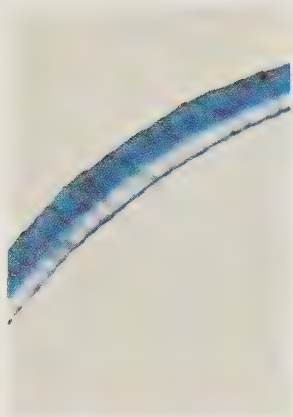


Fig. 2

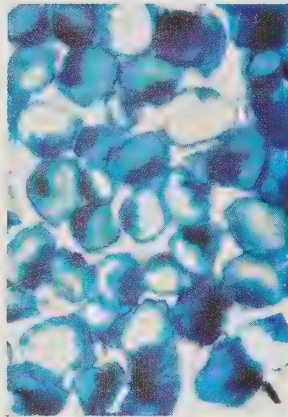


Fig. 5

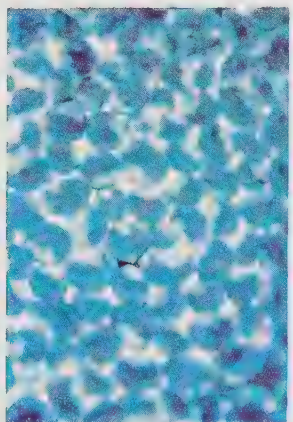


Fig. 1

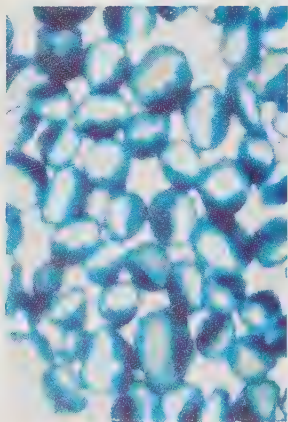


Fig. 4

BILATERAL STRUCTURE OF WOOL CORTEX

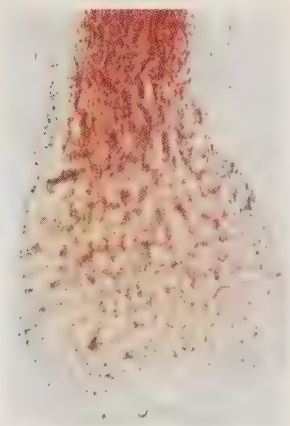


Fig. 3

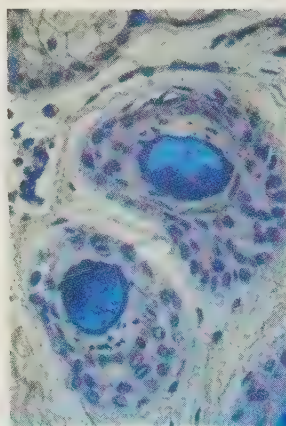


Fig. 6

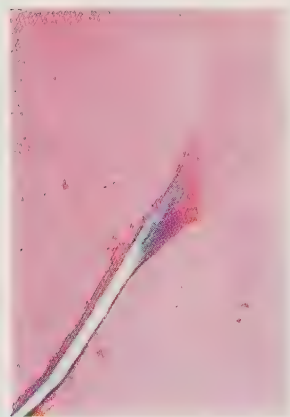


Fig. 2

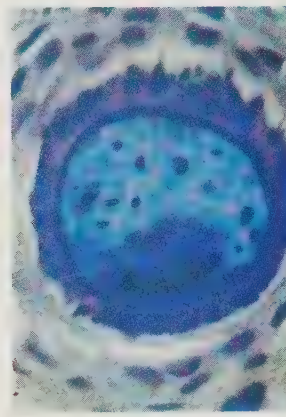


Fig. 5

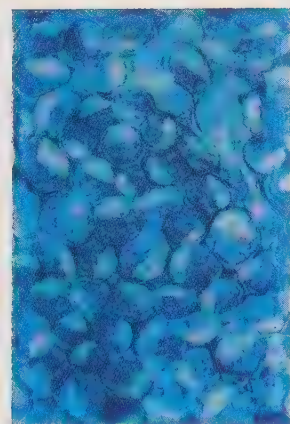


Fig. 1

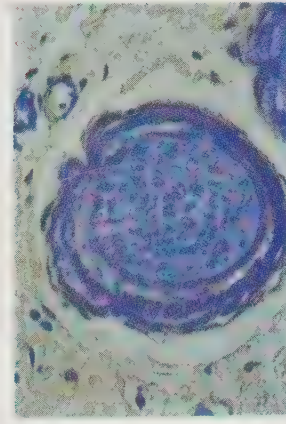
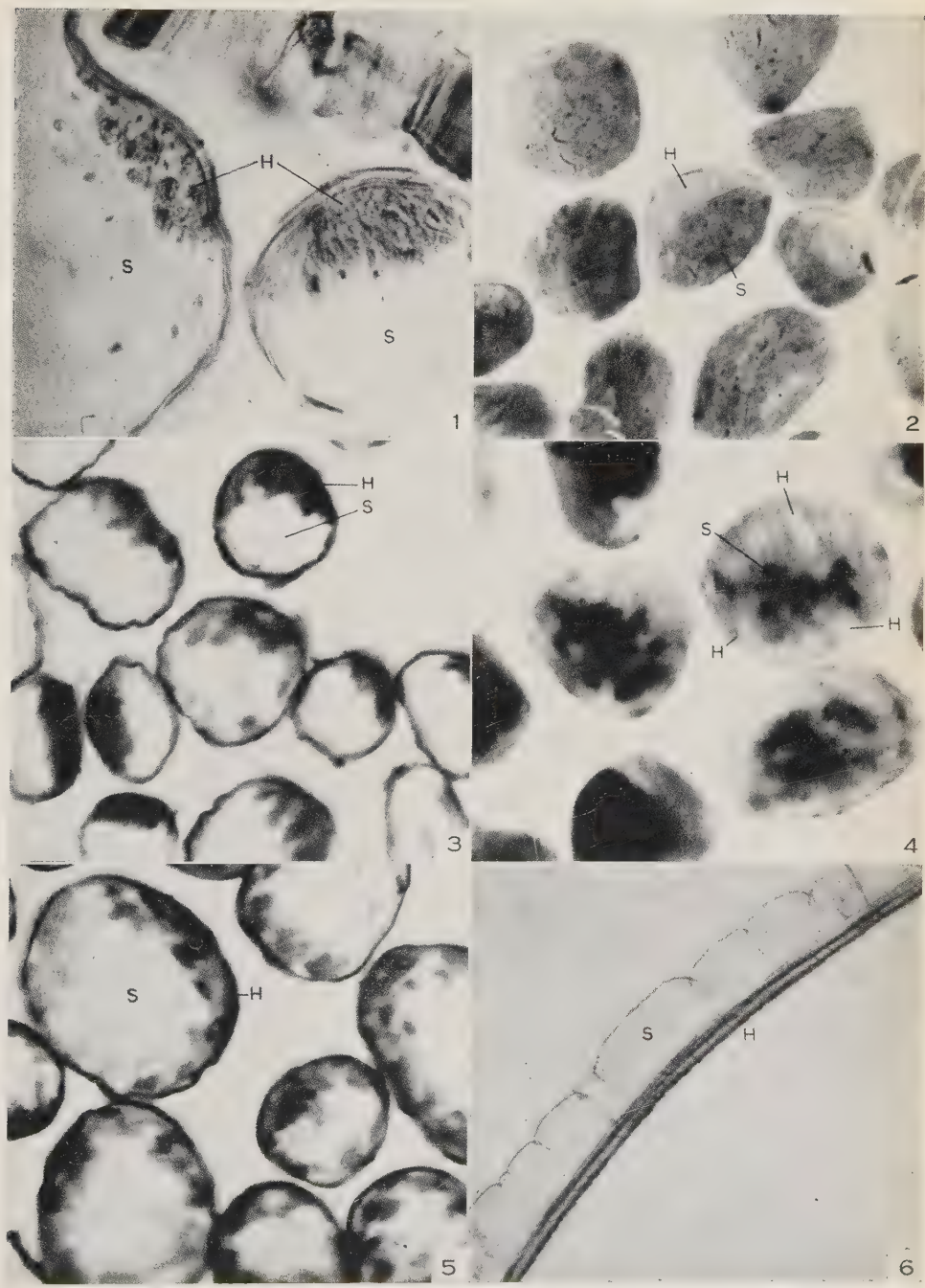
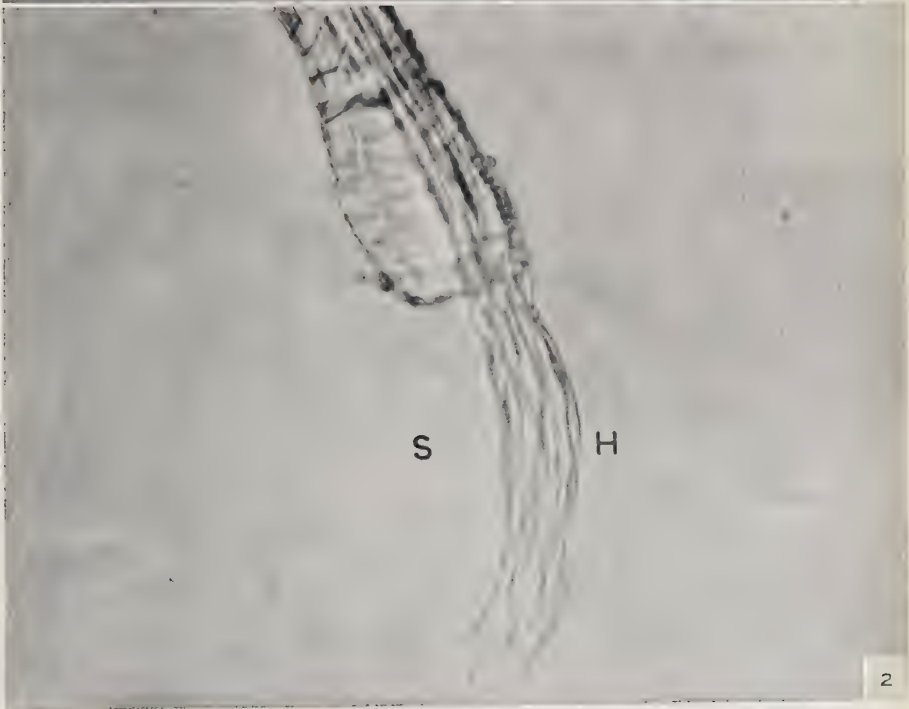
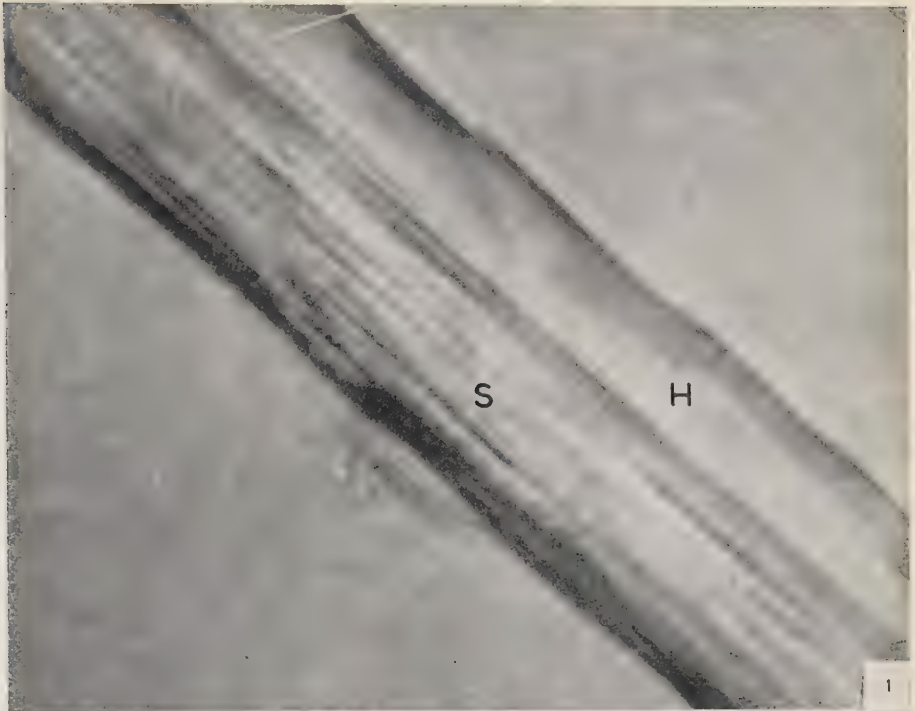


Fig. 4

BILATERAL STRUCTURE OF WOOL CORTEX



BILATERAL STRUCTURE OF WOOL CORTEX



NUTRIENT INTERACTIONS AND DEFICIENCY DIAGNOSIS IN THE LETTUCE

I. NUTRITIONAL INTERACTION AND GROWTH

By D. W. GOODALL,* A. E. GRANT LIPP,† and W. G. SLATER‡

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Summary

A sand-culture experiment with lettuces is described, having as its principal purpose the study of the relationship between the potential responses of plants to applications of nitrogen, phosphorus, and potassium fertilizers and the composition of their foliage. Plants were supplied initially with five levels of these nutrients in all combinations, samples of plant material were taken for analysis at various stages of development, and at 44 days from sowing additional quantities of nutrients were supplied to some of the cultures in order that their response potentialities might be determined. The present paper analyses the effects of the nutrient interactions on plant dry weight, further results being left to subsequent papers.

The most marked effects on growth were those of phosphorus supply, which resulted in increased dry weight throughout the range tested. Nitrogen supply gave an optimum-type curve, with a tendency for the optimum level to shift upwards with increasing phosphorus supply. The highest nitrogen supply level had a clearly adverse effect on growth. For potassium too there is a suggestion of an optimum-type curve, with an optimum shifting in accordance with phosphorus supply, but the plants were less sensitive to very considerable (30-fold) changes in potassium supply than in the case of either of the other nutrients. Within the sub-optimal range, the general effect of increasing the supply of one nutrient was to increase the proportional responses to the others. Calculation of relative growth rates indicated that the nutritional effects were spread through the growth period of the plants and not concentrated at any one stage of development.

The effects of nutrient applications at 44 days were in general conformity with the effects of initial applications. Plants whose initial treatment placed them in the sub-optimal range for a given nutrient, as indicated by the results from varying initial treatments, also responded, often very markedly, to a further application of this nutrient at 44 days.

The results of this experiment did not conform to the expectations from Mitscherlich's theories of the relation of plant growth to nutritional supply, but were in reasonable agreement with the "Resistance Formulae" of Maskell.

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I. INTRODUCTION

Much has been written in the past two decades on the use of chemical analysis of plant material as an aid in the diagnosis of mineral deficiency (see, for instance, Goodall and Gregory 1947). In most work on the subject, however, the purposes served have been qualitative. A disorder has been observed or suspected, and analysis of plant material has been performed in order to suggest or confirm a diagnosis of the disorder as due to deficiency of this or that nutritional element. In relatively few instances has there been an attempt to make the analytical data provide quantitative information as to the extent of the deficiency. Where "deficiency" is regarded as synonymous with the appearance of visual symptoms, there is, of course, no occasion for quantitative diagnosis by analysis—the quantitative indication is held to be provided by the severity of the symptoms. If, however, "deficiency" is considered to include any condition of a plant in which its "performance," however measured, suffers from the lack of a particular nutrient element and can be improved by an increase in its supply, no direct method of measuring the intensity of the deficiency is possible—there may well be no visually recognizable symptoms at all. If this meaning be ascribed to deficiency, then the most reasonable quantitative measure of deficiency is clearly the improvement in performance which may be expected to result from an increase in supply of the nutrient in question—a particular increase in supply which may be selected and specified arbitrarily. The resulting improvement in performance may be conveniently designated as "response," but can clearly be determined only by comparison between treated and untreated plants, and by allowing the disorder to run its course for at least one growing season. If chemical analysis could be used to estimate this potential response at an early stage in the plant's development, it would clearly be a much more valuable guide to fertilizer practice than any merely qualitative designation of deficiency.

Few investigators have attempted to establish a quantitative relationship between the response potentialities of plants and their chemical composition (e.g. Pfeiffer, Simmermacher, and Rippel (1919) with oats, van Itallie (1935) with grassland, Macy (1936) with barley, Crowther (1937) with cotton, Craig (1940) with sugar-cane, Lundegårdh (1941, 1943, 1951) with oats, and Goodall (1948, 1949) with wheat and barley). In most of these investigations, the material for analysis was collected *after*—often many months after—the treatment used to determine the response potentialities of the plants. Most commonly, fertilizer treatments were applied at or before the time of sowing; responses were estimated from the differences in yield between treated and untreated plants; and material from the untreated plants or those receiving a lower level of treatment, collected at or around harvest time, provided the analytical data. In other words, information required for a forecast was available only *after* an operation to whose effects the forecast referred. As a guide to fertilizer practice, such forecasting methods would, of course, be useless. But the rationale of these investigations was the expectation that the nutrient-supplying powers of the soil would remain reasonably similar from year to year,

and that information on the nutritional status of one crop would be valuable in determining the fertilizer regime for the next crop grown in the same field. That is, the analyses were envisaged as indicating the nutritional status of the plants analysed, not with a view to correcting any deficiencies detected in those plants, but in order that the correlations between their nutritional status on the one hand and that of the substrate and the plants subsequently to be grown in it on the other might enable the fertilizer regime of the latter to be adjusted.

In addition to being more immediately applicable in practice, the idea of analysing plants early in their development with a view to forecasting the response to a fertilizer treatment applied subsequently (e.g. by top-dressing or foliar application) seems theoretically more acceptable than the use of analyses of material collected at harvest-time. Some of the reasons for this have been mentioned in an earlier publication (Goodall and Gregory 1947); as they approach maturity, differences between plants in nutrient uptake tend to be apparent more in growth than in percentage composition; and it seems likely that the response of a plant to a nutritional treatment would be much more directly related to its composition at or before the time of application than to the composition of untreated plants several months later.

Partly in response to these considerations, interest has, in recent years, shifted from analysis of plant material at harvest-time as an index of soil conditions to analysis at earlier stages of development, with a view to using the information in improving the growth of those same plants. But very little of this work has been more than qualitative; the rapid "tissue tests" (see Nicholas 1953), for instance, have proved valuable in indicating whether plants are likely to respond to a nutrient addition during their development, but they have not been used to estimate the magnitude of these responses except in the broadest categories. It would appear that the only cases in which plant material has been analysed early enough in development for deficiencies still to be remediable, and in which the analyses could be used for quantitative forecasts of response, are in manganese deficiency of wheat (Goodall 1949) and possibly nitrogen deficiency in cotton (Crowther 1937).

The purpose of the present work was to obtain such data in respect of the major-element nutrition of lettuce. Apart from the first order relationships between the response to an element and the content in the plant of the same element, it was hoped to discover whether these relationships were affected by the status of the plant with respect to the other major nutrients. Lundegårdh (1941) found that the curve showing the relationship between the response of oat plants to potassium fertilizers and their potassium content varied with their phosphorus content—the higher the phosphorus content, the greater the response to potassium shown by plants of a given potassium content. He also found similar interactions between nitrogen response and phosphorus content, and between phosphorus response and nitrogen content. Generally, in work on nutritional diagnosis by plant analysis, it has been assumed that each nutrient can be treated independently. It was hoped to find whether, in the very different conditions of the present investigation, Lundegårdh's conclusions could be substantiated and perhaps extended.

It was first necessary to grow plants which, at an age of a few weeks, differed widely in nutritional status and in potential response to the three major nutrients — nitrogen, phosphorus, and potassium. Such plants could most readily be obtained by sand-culture methods. When they had reached a suitable stage, the responses were to be measured by supplying additional amounts of the same nutrients. Immediately before these treatments were applied, and on several earlier occasions, samples of plant material were to be taken for analysis. The analyses were to cover the major-element content of several different organs, and, in the case of nitrogen and phosphorus, different chemical fractions were to be estimated.

The results of such an experiment clearly have interest considerably beyond the questions which they were designed to answer. Information is obtainable from it on the effects of the nutrients and their interactions on the growth of the plants, on their nutrient uptake, and on their composition. Although the initial treatments were intended simply to provide plants differing widely in nutritional status, they included so many combinations as to provide more extensive material for the study of nutrient interactions than in many experiments designed expressly for that purpose. The first paper of this series will accordingly not deal with those parts of the results bearing directly on deficiency diagnosis, but will concern itself only with the effects of the treatments on plant growth, expressed in terms of dry weight. Subsequent papers will discuss the analytical results and their interpretation in relation to deficiency diagnosis.

It will be appreciated that the complete numerical data of a large and complex experiment cannot be presented in a printed paper. They are, however, recorded in full in the theses on which these papers are based (Grant Lipp 1952; Slater 1952), which may be consulted in the library of the University of Melbourne.

II. MATERIALS AND METHODS

The lettuce plants to be studied were grown in sand culture, under a cage of wire netting to prevent bird damage. The pots used were of glazed earthenware, fitted with a glass level tube, and each contained 6.7 kg of a mixture of white quartz sand and crushed quartz, thoroughly washed with tap and distilled water. They were arranged in double rows under glass shelters, in 125 groups of four. Each of these groups of four pots was allotted at random to one of the 125 initial treatments. By grouping the pots in this way, the effect of local differences in external conditions on the responses to subsequent nutrient additions was reduced, the measurement of these responses being one of the primary purposes of the experiment. These later additions, hereafter referred to as sub-treatments, were distributed randomly within each set of four pots.

The nutrient levels were chosen on the basis of results obtained in a preliminary experiment. The lowest levels (N_1 , P_1 , K_1) were those at which the plants would barely survive, the highest (N_5 , P_5 , K_5) were, judging by these preliminary studies, in excess of the requirements for optimal growth.

The amounts supplied per pot were as follows:

Nutrient level	1	2	3	4	5
N as NaNO_3 (mg)	50	200	500	2000	5000
P as Na_2HPO_4 (mg)	2	5	20	100	500
K as K_2SO_4 (mg)	0	100	300	1000	3000

Each pot also received 1200 mg of calcium as chloride and 600 mg of magnesium as sulphate. All the above were added in solution before the seeds were sown. In addition, each pot received a weekly supply of 2 mg of iron in the form of a solution of ferric citrate, and a trace element solution applied once a fortnight contained the following:

B as H_3BO_3	..	110 μg
Mn as MnSO_4	..	550 μg
Cu as CuSO_4	..	65 μg
Zn as ZnSO_4	..	25 μg
Mo as H_2MoO_4	.	10 μg

The sub-treatments (S_N , S_P , S_K) were applied 46 days after planting, the amount supplied in each case being equal to that in the lowest initial treatment level at which growth was satisfactory at this stage (N_2 , P_3 , and K_2), i.e.

S_N : 200 mg N per pot,
S_P : 20 mg P per pot,
S_K : 100 mg K per pot.

All nutrient solutions were prepared from analytical reagent grade chemicals and distilled water. Distilled water was also used to maintain the solution at a level approximately 15 cm below the surface of the sand.

The seeds of lettuce, variety Great Lakes, were sown on October 31, 1949, each pot receiving five groups of about ten seeds. At 11 days the seedlings were thinned to three in each of the five groups, and at 22 days to one in each group. The seedlings removed during these thinning processes provided the first two harvests; late-germinated seedlings were rejected, but among the others those to be retained on each occasion were selected at random. These first two harvests included only those 27 treatments combining the 1, 3, and 5 levels of each nutrient. On subsequent occasions, plants were harvested from all 125 treatments; at the third (29 days) two plants were taken from each pot, at the fourth (37 days), fifth (44 days), and sixth (98 days) harvests one only. The plants removed for harvesting were brushed clean, and the roots washed. In the first five harvests, before the sub-treatments had been applied, the plants from the four replicate pots were combined into two groups for weighing and analysis, each group representing a pair of pots.

In the first harvest, plants were dried and weighed intact, but in subsequent harvests the roots were cut off at the cotyledonary node, the fresh weight of the shoot was determined, and in some cases certain organs were separated from the shoot before drying. In the 22-day harvest, the cotyledons were separated in this way; in the 37- and 44-day harvests, the tops were divided into younger leaves, laminae of older leaves, and midribs plus stems, in so far as the

size of the plants made it possible. All samples were enclosed in paper bags and dried at 85-95°C.

TABLE 1
ANALYSES OF VARIANCE: DRY WEIGHT

Days from Sowing	Source of Variation	Degrees of Freedom	Mean Square	F
11†	N	2	0.3750	3.87
	P	2	0.1376	1.42
	K	2	1.5448	15.96**
	N × P	4	0.3314	3.42
	N × K	4	0.0987	1.02
	P × K	4	0.1010	1.04
	N × P × K	8	0.0968	
	Error	(23)	(0.0572)	
22†	N	2	82.498	7.76*
	P	2	187.233	17.60**
	K	2	97.889	9.20**
	N × P	4	16.370	1.54
	N × K	4	10.507	—
	P × K	4	16.641	1.56
	N × P × K	8	10.638	
	Error	(27)	(2.988)	
29‡	N	4	0.8318	16.31***
	P	4	3.0737	60.27***
	K	4	1.1867	23.27***
	N × P	16	0.1373	2.69**
	N × K	16	0.0488	—
	P × K	16	0.1382	2.71**
	N × P × K	64	0.0510	
	Error	(125)	(0.0289)	
37‡	N	4	0.7454	8.91***
	P	4	8.3978	100.33***
	K	4	0.7975	9.53***
	N × P	16	0.1629	1.95*
	N × K	16	0.0884	1.06
	P × K	16	0.2111	2.52**
	N × P × K	64	0.0837	
	Error	(125)	(0.0525)	
44‡	N	4	0.8649	9.27***
	P	4	12.1716	130.46***
	K	4	1.3946	14.95***
	N × P	16	0.2066	2.21*
	N × K	16	0.0853	—
	P × K	16	0.1805	1.93*
	N × P × K	64	0.0933	
	Error	(125)	(0.0740)	

TABLE 1 (*continued*)

Days from Sowing	Source of Variation	Degrees of Freedom	Mean Square	<i>F</i>
98§†	N	4	0.931	4.39**
	P	4	15.816	74.60***
	K	4	1.432	6.75***
	N × P	16	0.540	2.55**
	N × K	16	0.147	—
	P × K	16	0.221	1.04
	N × P × K	62	0.212	

P* = 0.01-0.05.*P* = 0.001-0.01.****P* < 0.001.

† Untransformed values.

‡ Logarithmic transformation.

§ Pots without sub-treatment only.

In view of the considerable differences in size at 44 days between plants subjected to the same treatments, and the importance attached to accurate estimation of the effects of the sub-treatments which were then about to be applied, the length and breadth of the largest leaf of each plant harvested at this time and of the remaining plant in each pot were measured; these measurements enabled analysis of covariance to be applied to the sub-treatment effects.

When the final harvest of all remaining plants was taken at 98 days, hearting was already well in progress in the best grown plants, though commercial maturity had not yet been reached.

III. STATISTICAL TREATMENT OF DATA

(a) *Analysis of Variance*

Except in the first harvest, the main analysis of the results was conducted on dry weights of aerial parts only, the root weight data being considered less reliable through losses in removing from the pots. These dry weight data were subjected to analysis of variance. In the harvests from 29 days onwards it was found that the differences between duplicate weights were nearly proportional to their means. The logarithmic transformation was accordingly applied, though this tends to over-correct at the highest weights. For the first two harvests with data from only 27 treatments the untransformed weights were used, since here the dependence of variance on mean was slight.

At each harvest the variances due to each of the three initial treatment series and their first and second order interactions were separated. A "within-treatments" error term was calculated, but could not be used in testing the significance of main effects and interactions, because a position effect was confounded with them on account of the non-random distribution of the replicate pots. The N × P × K interaction was used instead in testing for significance. The results of analysis of variance at each harvest date are shown in Table 1.

In the analysis of sub-treatment effects at 98 days, the final shoot dry weights have been corrected for differences in size at the time the sub-treatments were applied. In order to do this, an analysis of covariance was performed, the dependent variable being log dry weight at 98 days, and the independent variable log (product of length and breadth of largest leaf at 44 days).

(b) *Missing Values*

In its final stage the experiment comprised 500 different treatments and sub-treatments, without replication. Of these 500 plants 97 died before the final harvest, of which 16 were lost by accident or fungal attack, while the remaining 81 died without overt signs of pathogenic disease, probably as a result of the nutritional treatment to which they had been subjected (most were in the low-phosphorus treatments). It was necessary to replace these missing values before proceeding to the analysis of covariance.

Since the 81 plants dying as a presumed result of the nutritional treatments were very small, and their growth had appeared stationary since the harvest at 44 days, it was assumed that their dry weights had not increased appreciably during this period; accordingly, their weights at 44 days were estimated, and were used in the analysis for 98 days. These estimates were calculated from a regression on the measurements intended for the final analysis of covariance. Computing the regression was made more difficult by the fact that the plants had been paired before weighing. Since in this way only the mean weight of each pair was available for relating to the individual leaf measurements, and a linear regression seemed unsuitable, only pairs in which the two plants were reasonably similar in size could be used in estimating the regression. The standard of similarity arbitrarily chosen was such that the weight of the larger plant was not more than one and a half times that of the smaller. Of the 250 pairs of values, 87 met this requirement. Of various possible regressions of plant dry weight on leaf measurements that were tested, the linear regression of the logarithm of dry weight on that of the product of length and breadth of the largest leaf was found to give the most satisfactory results. This regression was highly significant ($r = 0.995$; $n = 85$) and led to the following expression

$$\log (W_1 + W_2) = 0.593 + 1.100 \log (L_1 B_1 + L_2 B_2),$$

where W_1 , W_2 are the weights of the two plants, and L_1 , L_2 , B_1 , B_2 the lengths and breadths of their largest leaves. If the two plants are assumed to be equal in size and weight, this reduces to

$$\log W = 0.623 + 1.100 \log LB,$$

for one plant. This method of estimating dry weight was used only when the loss of the plant could reasonably be ascribed to nutrient deficiency, but not when the plant had grown to any extent after 44 days or when a sub-treatment had been applied which would have been expected to induce a response. As the estimates were based on the dimensions of the same plants, no degrees of freedom were deducted for them in the analysis of variance.

The method used to replace missing values for the other 16 plants was the one, of several tried, which gave the closest fit when applied to existing

values. The regression of $\log W$, 98 days, on $\log LB$, 44 days, was calculated for the 101 surviving plants which received no sub-treatment. From this regression the calculated dry weight, based on the leaf dimensions, was found for each of the 403 surviving plants. The differences between the actual dry weights and those calculated from the regression were the variates used in fitting the 16 missing values. The mean difference was found for each level of N, P, and K in each of the four sub-treatment groups; interactions among

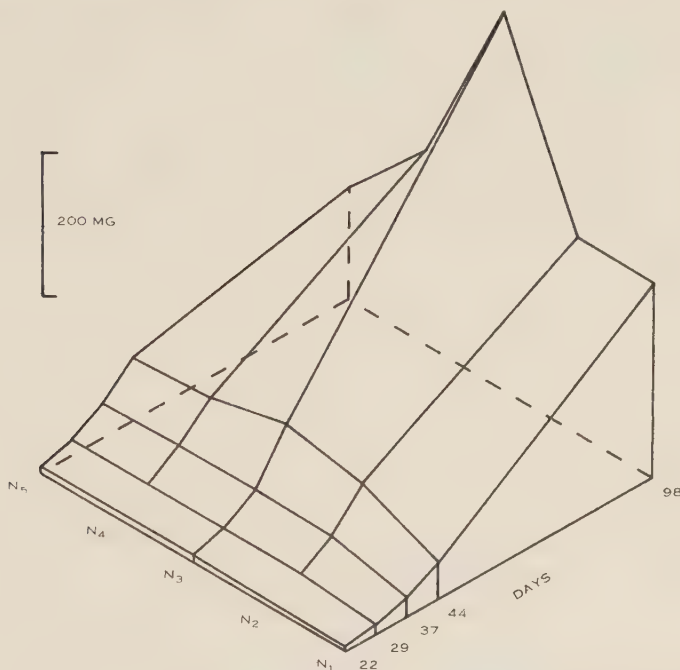


Fig. 1.—Effect of nitrogen supply on dry weight at different stages of development (logarithmic means except at 22 days).

the initial treatments were not taken into account. There were thus 60 means ($3 \times 5 \times 4$) each based on 25 values, less any estimated or missing values. The fitted value was obtained by adding to the calculated dry weight (from the regression equation) a correction term based on the mean of the three relevant mean differences. For example, the correction term for the treatment $N_3P_1K_2$, without sub-treatment, was the mean of the mean differences for N_3 , P_1 , and K_2 , all without sub-treatment. One degree of freedom was subtracted for each of these fitted values in the analysis of variance.

IV. RESULTS

(a) Appearance and Dry Weight as Affected by Initial Treatments

(i) *Nitrogen Initial Treatments.*—Plants receiving the treatments N_1 and N_2 were small and generally pale. Yellowing of the leaves was evident within

a week from emergence, and after a month was most marked at the tips of the older leaves. Hearts were not formed by N_1 plants. The mean dry weight for each nitrogen treatment at each harvest is shown in Table 2; the significant differences* quoted, together with the relevant entries in the analyses of variance (Table 1), enable the significance of the various effects to be assessed. To facilitate consideration of these effects, they are presented in the form of a solid diagram in Figure 1.

The dry weights of the whole plants at 11 days showed a distinct, but non-significant increase over the range N_1 - N_3 - N_5 . At later harvests, however, the treatment N_5 appeared to be supra-optimal. At 29 days there was no significant change in dry weight over the range N_2 - N_5 . At 37 days the increase between N_1 and N_2 was followed by a smaller increase up to N_3 - N_4 , and a decline at N_5 . This trend was continued at 44 days, when the maximum was again reached at N_3 , and the decline to N_5 was more marked. The dry weights at 98 days also showed a maximum at N_3 , and here the decline between N_4 and N_5 was very substantial.

TABLE 2
EFFECT OF NITROGEN SUPPLY ON DRY WEIGHT (MG)

Nitrogen Supply Level	Days from Sowing					
	11	22	29*	37*	44*	98*
1	1.7	4.1	12	27	54	277
2	—	—	23	50	101	278
3	1.8	7.7	23	53	122	538
4	—	—	24	52	97	277
5	2.0	7.9	23	49	90	153
Significant difference	0.34	3.55	—	—	—	—
Significant ratio	—	—	1.34	1.46	1.49	1.82

* Logarithmic means.

(ii) *Phosphorus Initial Treatments.*—The visual effects of phosphorus deficiency were not evident as early as those of nitrogen or potassium, but differences in size were apparent a fortnight after emergence. After 4 weeks, plants from treatments P_1 and P_2 were yellow, but the characteristic red and purple tints usually associated with phosphorus deficiency (e.g. Wallace 1951) were not observed. In many cases, however, the outer leaves were withered. Except for a few P_3 plants, those with phosphorus supply below P_4 did not heart. At later stages, most of the plants with low phosphorus supply showed little or no growth, and many died.

* Based here, as in Tables 3 and 4, on the $N \times P \times K$ interaction, the replication being ignored.

In each harvest from 22 to 44 days, increased phosphorus supply resulted in a marked increase in dry weight over the whole range P_1 - P_5 (Table 3, Fig. 2). In the 98-day harvest, however, the increase from P_4 to P_5 was negligible. In general, increases in growth with increasing phosphorus supply were more substantial than for either of the other nutrients — at 44 days, for instance, the mean dry weight for P_5 was nearly 14 times that for P_1 , while corresponding ratios for nitrogen and potassium were in each case about 3.

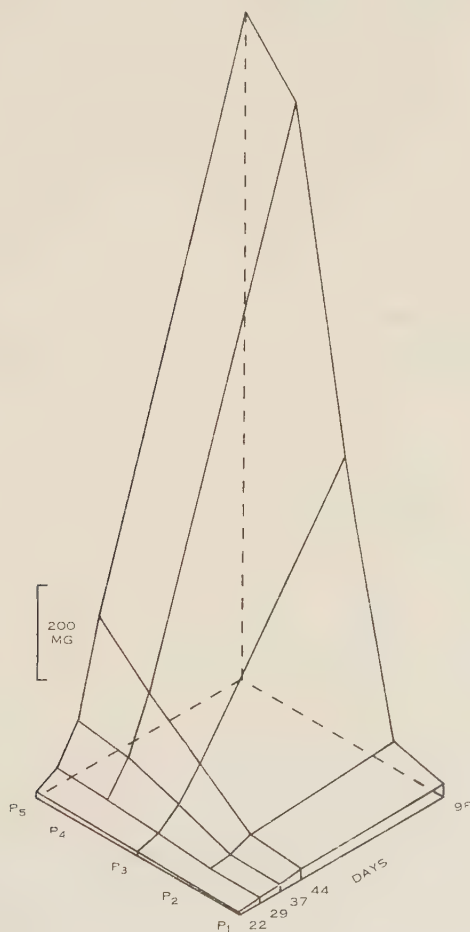


Fig. 2.—Effect of phosphorus supply on dry weight at different stages of development (logarithmic means except at 22 days).

(iii) *Potassium Initial Treatments.*—Within one week from emergence, plants from K_1 , and to a smaller extent from K_2 , showed slower growth than those from higher potassium levels. After 1 month several features which have apparently not been described previously for potassium-deficient plants were evident. The leaves were thick and rounded, in some cases heart-shaped, becoming smoother and rather fleshy when older. The margins were flat and

almost entire while those of normal plants were crinkled and indented. The effect on colour was not very marked, but generally the leaves were a dull, dark green with paler midribs and veins. During later stages of growth the leaves tended to become hook-shaped, as if by increased growth of the midrib. The stem bases were thickened and root systems extremely poor. Plants from K_1 did not heart.

Potassium deficiency affected the rate of growth visibly, before deficiency of either of the other nutrients, and this is again shown in the analysis of variance of nutrient effects on dry weight (Tables 1 and 4, Fig. 3). At 11 days the effect of potassium on the untransformed dry weights of whole plants was highly significant ($P < 0.01$), although none of the other treatment or interaction effects reached the 5 per cent. level. In all subsequent harvests the plants receiving no potassium (K_1) were considerably smaller than the others, but among the latter, differences were less marked and rather irregular. In the final harvest there is a suggestion of an optimum at K_3 .

TABLE 3
EFFECT OF PHOSPHORUS SUPPLY ON DRY WEIGHT (MG)

Phosphorus Supply Level	Days from Sowing					
	11	22	29*	37*	41*	98*
1	1.8	3.5	10	15	24	28
2	—	—	12	18	32	57
3	1.7	6.3	24	61	120	600
4	—	—	30	97	206	1295
5	1.9	9.9	38	114	311	1422
Significant difference	0.34	3.55	—	—	—	—
Significant ratio	—	—	1.34	1.46	1.49	1.82

* Logarithmic means.

(iv) *Interactions of Initial Treatments.*—The principal interest in these data centres in the interaction effects between the three nutrients. From Table 1 it will be seen that there is a significant interaction between phosphorus and nitrogen in all harvests from 29 days onwards and between phosphorus and potassium at 29, 37, and 44 days.

The nitrogen-phosphorus interactions are illustrated in Figure 4, which shows in the form of solid diagrams the mean dry weight for each nitrogen-phosphorus combination at each harvest. A diagram for the 22-day harvest is included, the effect being already in evidence at this stage, though not yet significant. An increase in the nitrogen supply from N_1 to N_2 increases the proportional response to phosphorus at all harvests and from 44 days onwards a further rise in the response to phosphorus can be seen when the nitrogen supply is

increased to N_3 ; the latter rise, however, occurs only at the upper phosphorus levels—the proportional increase in yield due to increase in phosphorus supply up to P_3 is unaffected by nitrogen above N_2 . The increase in dry matter with increase in phosphorus supply occurs throughout the range if the nitrogen supply is adequate, but if not (i.e. at N_1) dry matter increases only up to P_3 .

If one now considers the response to nitrogen at different levels of phosphorus, one notices that at the lowest level (P_1) the dry matter yield varies only slightly with nitrogen supply. At higher phosphorus levels the responses to nitrogen become progressively more marked. This is true both of the positive responses up to N_3 or N_4 and of the negative responses when this optimum is exceeded. The optimum nitrogen level does not seem to depend greatly on the phosphorus supply. The magnitude of the interaction effects, like those of the mean responses, tended to increase as the plants grew older.

TABLE 4
EFFECT OF POTASSIUM SUPPLY ON DRY WEIGHT (MG.)

Potassium Supply Level	Days from Sowing					
	11	22	29*	37*	44*	98*
1	1.5	4.0	11	27	46	111
2	—	—	22	51	110	283
3	1.9	7.1	22	52	99	465
4	—	—	28	56	119	380
5	2.0	8.6	23	46	98	316
Significant difference	0.34	3.55	—	—	—	—
Significant ratio	—	—	1.34	1.46	1.49	1.82

* Logarithmic means.

Figure 5 illustrates the interactions between phosphorus and potassium supply. In the absence of added potassium (K_1) dry weight increased with increasing phosphorus supply only up to P_3 . At the potassium levels K_2 and K_3 , the increase continued up to P_4 and in later harvests to P_5 . At the highest potassium levels, increased phosphorus supply caused increase in yield throughout the range even at 29 days.

At any given level of phosphorus supply, the maximum dry weight usually occurred at an optimum intermediate level of potassium supply, this optimum being somewhat displaced according to the phosphorus supply. At the lower levels of phosphorus, K_3 generally represented the optimum; for higher levels it was K_4 .

The nitrogen-potassium interaction is nowhere significant if tested rigorously against the second order interaction; but the variance due to it was significantly greater than that between replicates (which, as already explained, did not

involve any position effect) at 22, 29, and 37 days. Since at each harvest the interaction is in the same direction, it may probably be regarded as meaningful; it is shown in the solid diagrams of Figure 6. The proportional increase in dry weight with increasing potassium supply is greater at higher levels of nitrogen supply. This is particularly true if N_1 and K_1 are compared with the means of N_2-N_5 and K_2-K_5 as is shown by the values in Table 5.

No rigorous test of the significance of the second order interaction is possible, since it is confounded with a position effect, probably small; but the two together are significantly greater than the variance between replicates at 22, 29, and 37 days. This second order interaction takes the form of a reinforcement of first order interactions at favourable levels of the third factor. Consider, for instance, that part of the interaction between phosphorus and potassium, which is represented by

$$(K_3 P_5 - K_1 P_5) - (K_3 P_1 - K_1 P_1).$$

The values for this interaction element at successive harvests for different levels of nitrogen supply are shown in Table 6.

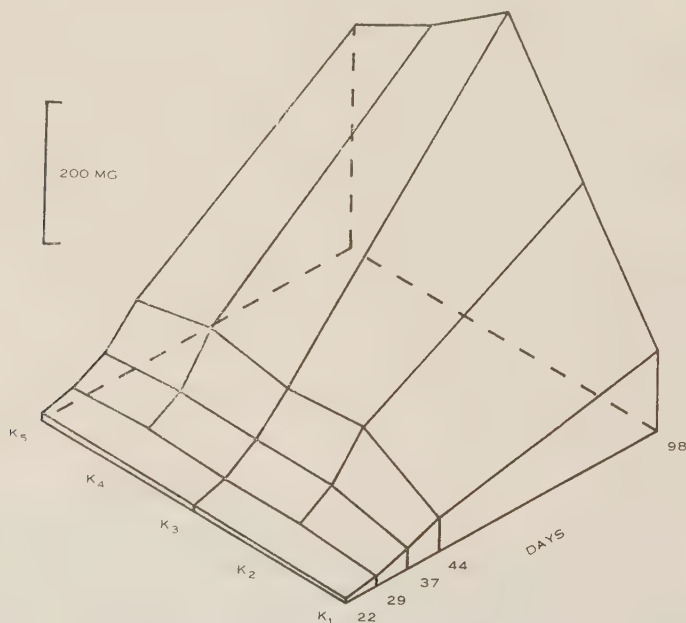


Fig. 3.—Effect of potassium supply on dry weight at different stages of development (logarithmic means except at 22 days).

(b) Relative Growth Rate as Affected by Initial Treatments

Relative growth rates (R.G.R.) were calculated for each of the periods from 11 to 44 days, for the 27 treatments in which data for all these harvests were available. For this purpose, of course, dry weights of the whole plant were required, and it was therefore necessary to include the rather unsatisfactory root dry weight data—the contribution of which was, however, comparatively

small. These growth rates were subjected to analysis of variance, and the results are presented in Tables 7 and 8. It will be noted that the mean R.G.R. was highest in the earliest period, and then fell to a steady level.

TABLE 5
MEAN PERCENTAGE INCREASE IN DRY WEIGHT WITH
POTASSIUM SUPPLY AT DIFFERENT NITROGEN LEVELS

Days from Sowing	N ₁	N ₂ -N ₅
11*	48	32
22*	66	118
29	37	138
37	23	109
44	42	162
98	60	278

* Levels 1, 3, 5 only.

Increase in nitrogen supply from N₁ to N₃ appears to have increased R.G.R. during most periods, although the effect did not at any stage reach significance. Above this level, there was little change.

From 11 to 22 days, potassium was the only nutrient having a significant effect on R.G.R. When its supply was raised from K₁ to K₃, there was a substantial rise in R.G.R., but further increase did not affect it. The effect of

TABLE 6
P × K INTERACTION AT DIFFERENT NITROGEN LEVELS (SEE TEXT)

Nitrogen Level	Days from Sowing					
	11*	22*	29†	37†	44†	98†
N ₁	-0.29	-3.58	-0.44	-0.10	-0.57	-1.55
N ₂	—	—	0.47	0.58	0.11	0.41
N ₃	0.15	8.59	0.78	0.48	0.75	0.53
N ₄	—	—	0.83	0.55	1.14	1.15
N ₅	1.04	8.61	0.59	1.27	0.74	0.89

* Absolute weights.

† Log weights.

potassium persisted during subsequent development, though it did not reach significance.

After 22 days the place of potassium as the important factor controlling R.G.R. was taken by phosphorus. Except in one instance, increase in phos-

TABLE 7
RELATIVE GROWTH RATE (PER CENT. PER DAY)

Nitrogen Levels		N ₁				N ₃				N ₅				Mean N ₁ -N ₅			
Days from Sowing	Phosphorus Level Potassium Level	P ₁	P ₃	P ₅	Mean P ₁ -P ₅	P ₁	P ₃	P ₅	Mean P ₁ -P ₅	P ₁	P ₃	P ₅	Mean P ₁ -P ₅	P ₁	P ₃	P ₅	Mean P ₁ -P ₅
11-22	K ₁	14	10	14	12.7	11	15	12	12.7	12	13	15	13.3	12.3	12.7	13.7	12.9
	K ₃	17	13	12	14.0	17	17	20	18.0	14	18	22	18.0	16.0	16.0	18.0	16.7
	K ₅	15	16	15	15.3	19	19	22	20.0	17	15	20	17.3	17.0	16.7	19.0	17.6
22-29	Mean K ₁ -K ₅	15.3	13.0	13.7	14.0	15.7	17.0	18.0	16.9	14.3	15.3	19.0	16.2	15.1	15.1	16.9	15.7
	K ₁	14	12	15	13.7	11	15	8	11.3	10	15	12	12.3	11.7	14.0	11.7	12.4
	K ₃	14	10	18	14.0	11	18	20	16.3	12	12	21	15.0	12.3	13.3	19.7	15.1
29-37	K ₅	13	12	15	13.3	9	20	18	15.7	11	13	23	15.7	11.0	15.0	18.7	14.9
	Mean K ₁ -K ₅	13.7	11.3	16.0	13.7	10.3	17.7	15.3	14.4	11.0	13.3	18.7	14.3	11.7	14.1	16.7	14.1
	K ₁	6	15	9	10.0	6	15	14	11.7	6	7	6	6.3	6.0	12.3	9.7	9.3
37-44	K ₃	2	18	9	9.7	10	13	14	12.3	-2	18	16	10.7	3.3	16.3	13.0	10.9
	K ₅	-3	6	4	2.3	6	-2	12	5.3	9	12	10	10.3	4.0	5.3	8.7	6.0
	Mean K ₁ -K ₅	1.7	13.0	7.3	7.3	7.3	8.7	13.3	9.8	4.3	12.3	10.7	9.1	4.4	11.3	10.4	8.7
	K ₁	-2	7	14	6.3	3	4	16	7.7	1	14	17	10.7	0.7	8.3	15.7	8.2
	K ₃	7	2	12	7.0	-2	6	19	7.7	13	9	12	11.3	6.0	5.7	14.3	8.7
	K ₅	7	14	13	11.3	16	16	22	18.0	0	18	12	10.0	7.7	16.0	15.7	13.1
	Mean K ₁ -K ₅	4.0	7.7	13.0	8.2	5.7	8.7	19.0	11.1	4.7	13.7	13.7	10.7	4.8	10.0	15.2	10.0

phorus supply resulted in a rise in R.G.R. During the period from 22 to 29 days, two phosphorus interactions also reached significance. Of these, the meaning of the $N \times P$ interaction is far from obvious, and it may well be a chance effect. The $P \times K$ interaction is clearer. At K_1 , increase in the phosphorus

TABLE 8
RELATIVE GROWTH RATE: ANALYSIS OF VARIANCE

Days from Sowing	Source of Variation	Degrees of Freedom	Mean Square	<i>F</i>
11-22	N	2	20.6	4.05
	P	2	9.4	1.97
	K	2	55.8	10.97**
	$N \times P$	4	8.6	1.69
	$N \times K$	4	6.2	1.22
	$P \times K$	4	0.4	—
	$N \times P \times K$	8	5.1	—
22-29	N	2	1.6	—
	P	2	56.3	11.95**
	K	2	19.7	4.18
	$N \times P$	4	24.3	5.16*
	$N \times K$	4	6.0	1.27
	$P \times K$	4	20.4	4.33*
	$N \times P \times K$	8	4.7	—
29-37	N	2	14.4	—
	P	2	126.4	7.02*
	K	2	56.2	3.12
	$N \times P$	4	26.6	1.48
	$N \times K$	4	31.2	1.73
	$P \times K$	4	29.0	1.61
	$N \times P \times K$	8	18.0	—
37-44	N	2	21.8	—
	P	2	245.5	10.98*
	K	2	65.8	2.94
	$N \times P$	4	21.9	—
	$N \times K$	4	32.2	1.44
	$P \times K$	4	31.2	1.40
	$N \times P \times K$	8	22.4	—

* $P = 0.01-0.05$.

** $P = 0.001-0.01$.

supply had no effect on R.G.R., but at K_3 and K_5 plants receiving the highest phosphorus supply (P_5) had a higher R.G.R. than those at an intermediate level (P_3). Increase in potassium supply increased R.G.R. only when phosphorus was abundant.

(c) Sub-Treatment Effects

The effects of the treatments applied at 44 days were studied on the basis of the analysis of covariance, using the logarithm of shoot dry weight at 98 days as the dependent and the logarithm of leaf dimensions at 44 days as the inde-

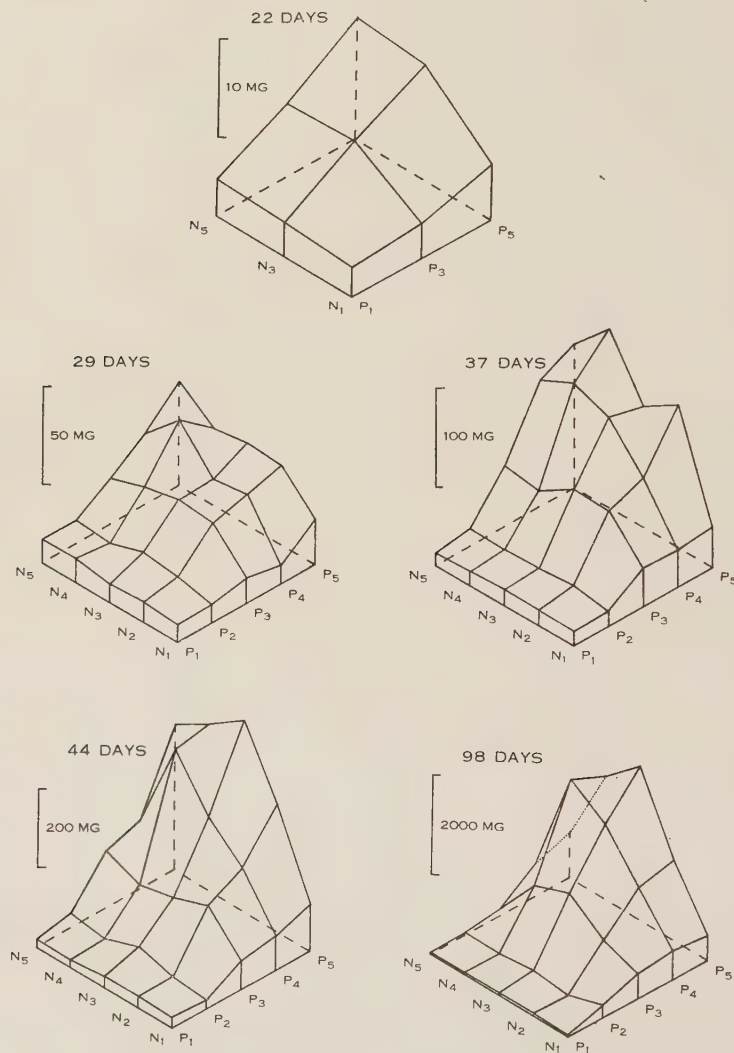


Fig. 4.—Interaction effects of nitrogen and phosphorus supply on dry weight (logarithmic means except at 22 days).

pendent variable. Since analysis of the data as a whole would not have led to a division of the degrees of freedom conveniently displaying the effects of each of the three sub-treatments in comparison with the common control, the data have been analysed in three separate portions, enabling these comparisons to

be made separately. But a common estimate of error was used in all three portions, based on the whole experiment. For this estimate the third order interaction between sub-treatments and main treatments ($S \times N \times P \times K$) was used, with 175 degrees of freedom; consideration of the independent variable reduced this error variance from 0.203 to 0.129.

As the quantities in these analyses are logarithms, the effects tested are the percentage responses. These were evaluated wherever they exceeded the significance level of 0.05, as follows:

$$\text{Percentage response} = \left\{ \text{antilog} \frac{(y_s - y_0) - b(x_s - x_0)}{n} - 1 \right\} \times 100,$$

where y_s is the total of n values of log dry weight at 98 days for sub-treatment S ,
 y_0 is the total of n values of log dry weight at 98 days without sub-treatment,

x_s is the total of n values of log product of leaf length and breadth at 44 days for sub-treatment S , and

x_0 is the total of n values of log product of leaf length and breadth at 44 days without sub-treatment.

The value of the regression coefficient b was obtained from the analysis of covariance for all sub-treatments: $b = 0.928$.

(i) *Nitrogen Sub-Treatment*.—The analysis of covariance is presented in Table 9, and the calculated values of some of the responses are given in Table 10. It may be seen that the average effect of the nitrogen sub-treatment was very small; this average conceals a fairly marked difference between a negative response at N_3 and N_4 and a positive response at N_1 and N_2 . The positive response at N_1 and N_2 was to be expected; the negative response at N_3 and N_4 must have been due to the total nitrogen supply reaching a toxic level, but as the sub-treatment (equal to the initial nitrogen supply in N_2) represented only a 4 per cent. increase in total nitrogen supply at N_5 , no further reduction in growth could be demonstrated here. This interaction between the nitrogen sub-treatment and the initial treatment just fails to reach significance.

A more definite interaction is found between the nitrogen sub-treatment and the initial phosphorus treatment ($S_N \times P$). A positive response of approximately 50 per cent. occurs at P_4 and P_5 ; at P_3 there is no response, and at the lowest phosphorus levels there is a negative response.

The responses to the nitrogen sub-treatment at different levels of initial nitrogen and phosphorus treatments become somewhat clearer when the second order interaction between all three factors ($S_N \times N \times P$) is considered. This interaction, though not reaching significance, indicates that the major positive responses to the nitrogen sub-treatment occurred in those cultures where initial nitrogen supply was low and phosphorus high. With other combinations of initial treatments, the response to nitrogen was usually small or negative—either because the nitrogen supply was already ample, or because the phosphorus status of the plants was too low to permit increased growth.

The only second order interaction of nitrogen sub-treatment with initial treatments which reached significance was that with phosphorus and potassium ($S_N \times P \times K$). Details of this interaction are also given in Table 10. Although there are several anomalous values in this table, the general trend is towards higher positive responses to nitrogen wherever the supply of phosphorus and potassium were both adequate for good growth (P_4 and P_5 , and K_2 - K_5). For example, there is no distinct trend at P_1 , but at P_5 there is a progressive increase in response from K_1 to K_5 .

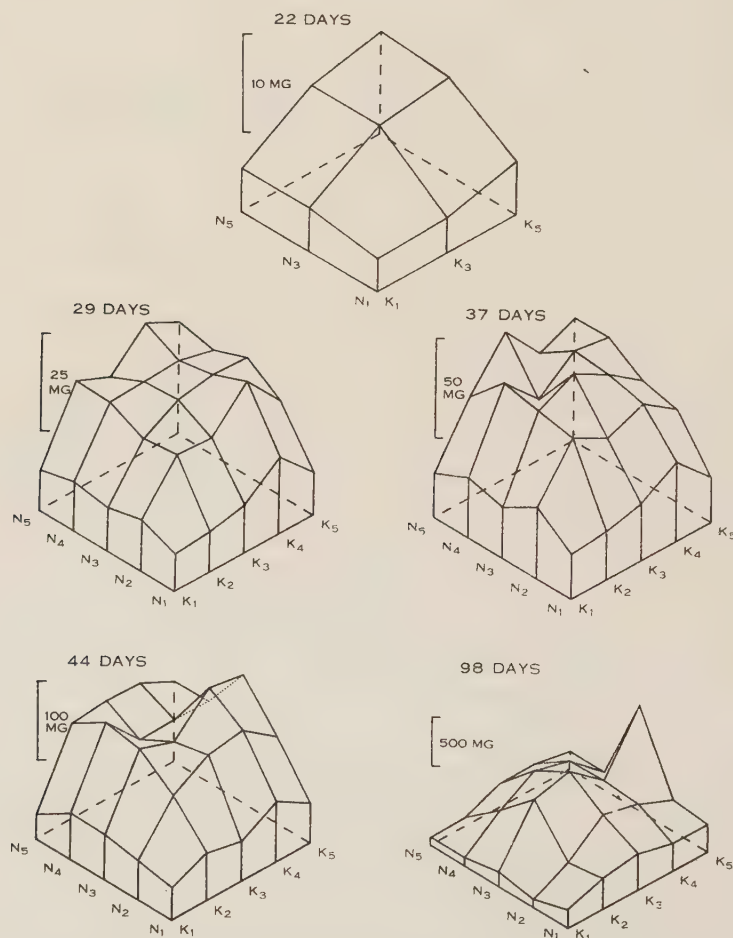


Fig. 5.—Interaction effects of phosphorus and potassium supply on dry weight (logarithmic means except at 22 days).

(ii) *Phosphorus Sub-Treatment*.—The overall effect of phosphorus sub-treatment is highly significant (S_P , Table 9), as also are the interactions with nitrogen initial treatment ($S_P \times N$) and with phosphorus initial treatment ($S_P \times P$). The significance of the second order interaction between phosphorus

sub-treatment and nitrogen and phosphorus initial treatments ($S_P \times N \times P$) almost reaches the 1 per cent. level. The calculated values of these responses are given in Table 10.

The effect of the sub-treatment was strongly dependent on the phosphorus initial treatment, the magnitude of the response declining steadily from P_1 to P_5 .

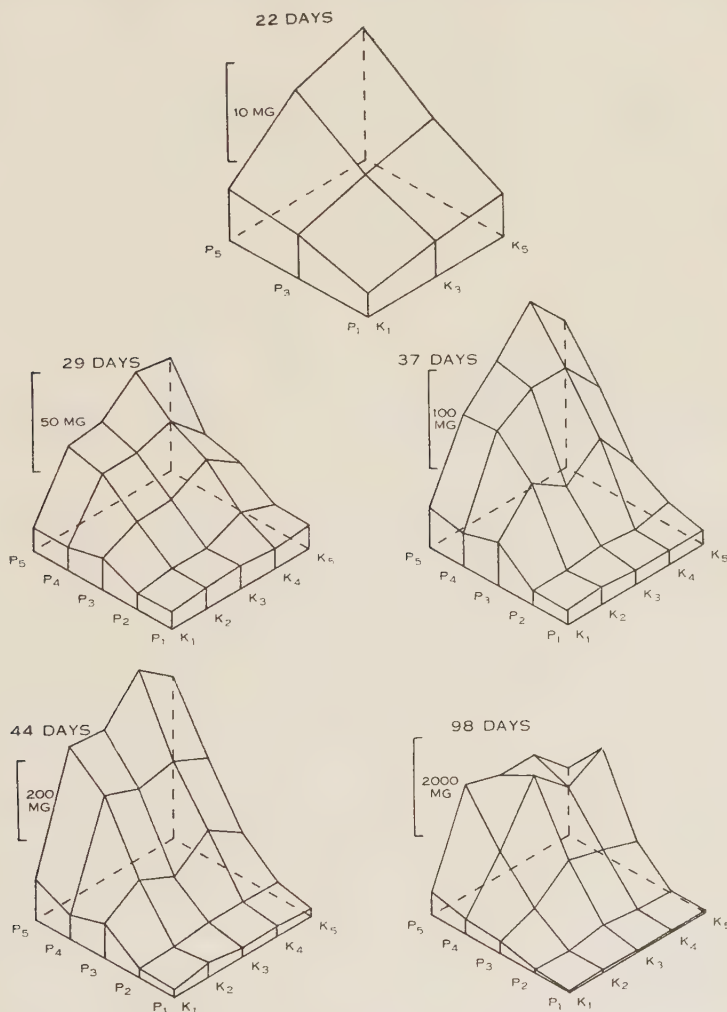


Fig. 6.—Interaction effects of nitrogen and potassium supply on dry weight (logarithmic means except at 22 days).

The significant interaction with nitrogen supply already mentioned depends on the difference between a small response to phosphorus at N_1 , and the much larger, although variable, responses at higher nitrogen levels. The effect may be specified more precisely from the second order interaction; generally the response was largest at the optimum levels of nitrogen supply (N_2 - N_4), although

at P_1 and P_2 there was a positive response at each nitrogen level. The responses at P_3 were small, and at P_4 and P_5 they were negligible. Inspection of the original data shows that over the range of initial treatments where yield was increasing as a result of increases in initial phosphorus supply, positive responses to the phosphorus sub-treatment were found. There is no evidence of adverse effects of excess within the range of phosphorus supply used in this experiment.

TABLE 9

SUB-TREATMENT EFFECTS: ANALYSIS OF COVARIANCE

All mean squares have been compared with the all-subtreatments error term to determine F . Degrees of freedom = 175; mean square = 0.129

Sub-Treatment	Source of Variation	Degrees of Freedom	Reduced Mean Square	F
Nitrogen	S_N	1	0.013	—
	$S_N \times N$	4	0.276	2.16
	$S_N \times P$	4	0.333	2.60*
	$S_N \times K$	4	0.016	—
	$S_N \times N \times P$	16	0.175	1.37
	$S_N \times N \times K$	16	0.082	—
	$S_N \times P \times K$	16	0.234	1.83*
Phosphorus	S_P	1	18.524	143.60***
	$S_P \times N$	4	0.744	5.76***
	$S_P \times P$	4	4.318	33.47***
	$S_P \times K$	4	0.097	—
	$S_P \times N \times P$	16	0.266	2.06*
	$S_P \times N \times K$	16	0.121	—
	$S_P \times P \times K$	16	0.122	—
Potassium	S_K	1	0.091	—
	$S_K \times N$	4	0.200	1.55
	$S_K \times P$	4	0.160	1.24
	$S_K \times K$	4	1.022	7.92***
	$S_K \times N \times P$	16	0.099	—
	$S_K \times N \times K$	16	0.099	—
	$S_K \times P \times K$	16	0.129	1.00

* $P = 0.01-0.05$.

** $P = 0.001-0.01$.

*** $P < 0.001$.

(iii) *Potassium Sub-Treatment*.—In this case only the interaction between sub-treatment and potassium initial treatment ($S_K \times K$) is significant (Table 9). The only definite response is at K_1 ; the others are small, and negative above K_2 . Although the other first order interactions are not significant, Table 10 shows that the response is positive at P_4 and P_5 , and negative at the lower phosphorus levels—a result reminiscent of the effect of phosphorus supply on nitrogen response mentioned above. There is no clear relationship with nitrogen supply.

V. DISCUSSION

The first point to be made in discussion is, perhaps, the physiological dissimilarity in the ranges tested for the three nutrients. In the case of nitrogen, there seems no doubt that the optimum supply, for the conditions of the experiment, was exceeded. The highest potassium supply was also, it would appear, in excess of that needed for maximum growth; and even where potassium was completely omitted from the nutrient solution (K_1) the plants could still make a reasonable amount of growth. But, for phosphorus, the optimum has barely been reached; at the higher nitrogen levels, there is still an increase in growth between P_4 and P_5 , and the effect of the phosphorus sub-treatment, though small, is positive. On the other hand, at the lower levels of phosphorus supply, the plants could barely survive, let alone grow. The phosphorus treatments were thus pitched at a lower physiological level than those of either of the other nutrients.

The marked adverse effects of high nitrogen doses, even where phosphorus supply was adequate, are noteworthy. Adverse effects on yield or quality of seed and fruit crops are commonplace, but in a purely vegetative crop like the lettuce it is more surprising. The optimum region for nitrogen would seem to be much more circumscribed than that for potassium, where the differences in growth over the range K_2 - K_5 were small although the supply varied by a factor of 30.

The data for R.G.R. do not assist greatly in the interpretation of the results. It must be remembered that they are based on less than a quarter of the results, so the lower significance levels than are seen in the analyses of dry weight data are not surprising. In general, they do not suggest that there is one stage of development more than another at which the nutrient effects on growth rate are particularly marked. The range of figures for R.G.R. appears much smaller than for the dry weight data, but since R.G.R. is a "compound interest" rate, and the differences persist in general throughout the period studied, this was to be expected. It is unfortunate that the data collected did not enable net assimilation rates to be calculated.

The responses to the sub-treatments at 44 days, though essential for correlating with the analytical data, do not in themselves add much to our knowledge of the nutrient status of the plants at that time. Where the effects of the initial treatments gave reason to suppose that a particular plant was suffering from deficiency of a nutrient, an additional supply of that nutrient increased its weight over that of the untreated pot; where there was already more than enough, an addition might depress growth.

These data provide interesting material for comparison with certain formal treatments of nutrient effects and interactions which are to be found in the literature. von Liebig's (1840) "Law of the Minimum," based on even less experimental evidence than Blackman's (1919) very similar Law of Limiting Factors in photosynthesis, postulated that the curve connecting yield with nutrient supply had a linear rising portion and an abrupt transition to a horizontal portion; the supply of another nutrient would affect only the point of transition.

This law has been long discredited in the form in which Liebig presented it (though one still often reads of a nutrient as being "in minimum"); and it fails to find support in the present data except perhaps as an extreme limiting

TABLE 10
PER CENT. RESPONSES TO SUB-TREATMENTS

*Nitrogen Sub-Treatment**

Average Effects at Different Levels of Initial Treatments

N ₁	+25	P ₁	-16	K ₁	-3
N ₂	+62	P ₂	-37	K ₂	+8
N ₃	-30	P ₃	-1	K ₃	-7
N ₄	-22	P ₄	+47	K ₄	+11
N ₅	+7	P ₅	+54	K ₅	+10

Interactions between Initial Treatments

	P ₁	P ₂	P ₃	P ₄	P ₅
N ₁	-4	-81	+8	+257	+345
N ₂	-30	+111	+58	+107	+130
N ₃	-45	-44	-17	-9	-32
N ₄	+25	-54	-57	+5	+11
N ₅	-12	-4	+56	-2	+11
K ₁	+24	-48	+115	-34	-9
K ₂	-67	-11	+63	+88	+7
K ₃	+43	-78	+19	+48	+27
K ₄	-49	-5	-27	+103	+132
K ₅	+38	+7	-69	+17	+196

Phosphorus Sub-Treatment†

Average Effects at Different Levels of Initial Treatments

N ₁	+36	P ₁	+1790	K ₁	+200
N ₂	+460	P ₂	+1020	K ₂	+260
N ₃	+290	P ₃	+130	K ₃	+200
N ₄	+410	P ₄	+16	K ₄	+240
N ₅	+250	P ₅	-7	K ₅	+390

Interactions between Initial Treatments

	N ₁	N ₂	N ₃	N ₄	N ₅
P ₁	+1470	+1470	+3830	+5230	+370
P ₂	+11	+3970	+1460	+1300	+1730
P ₃	-46	+370	+41	+280	+370
P ₄	-22	+100	+12	+3	+16
P ₅	-38	-10	-8	+15	+19

TABLE 10 (*continued*)
Potassium Sub-Treatment†
 Average Effects at Different Levels of Initial Treatments

N ₁	-16	P ₁	0	K ₁	+220
N ₂	+62	P ₂	-8	K ₂	+22
N ₃	-7	P ₃	-18	K ₃	-43
N ₄	-11	P ₄	+34	K ₄	-7
N ₅	+37	P ₅	+52	K ₅	-25

* Average overall effect = +3.

† Average overall effect = +250.

‡ Average overall effect = +9.

condition — the effects of increasing phosphorus supply when nitrogen or potassium is extremely low may approximate to the situation it implies.

Mitscherlich's (1909) formulation of the relationship between yield and nutrient supply has had much more consistent support, particularly in Europe and latterly in South America. This, as is well known, relates the gradient of the curve to the shortfall of the yield below an assumed maximum; it is a particular mathematical form of the Law of Diminishing Returns, so familiar in economics. In the present data, the curves representing effect of increase in the supply of a single nutrient, the others being held constant (i.e. the sections of the solid diagrams in Figs. 1-6), are in adequate agreement with Mitscherlich's representation of growth-nutrient relations, provided his later introduction of a "damage factor" is accepted (Mitscherlich 1928). But five points do not enable a satisfactory test of this method of dealing with the data to be made.

The most serious criticisms of Mitscherlich's view (see, for instance, Briggs 1925; Balmukand 1928) have been levelled against his treatment of nutrient interactions. He claimed that the "effect factor" was constant, irrespective of the supply of a second nutrient (and, indeed, of climatic environment, crop variety, or species, and any other factor which might influence the result). This claim has been disproved sufficiently frequently to need no further attention, were it not that, in certain quarters, much attention is still paid to Mitscherlich's work and the criticisms ignored. The present data may accordingly be used to buttress the opposition to his claims and to draw attention again to their inadequacy.

If Mitscherlich's effect factor were constant, the ratio of yields at two particular levels of phosphorus supply, say, would be independent of nitrogen supply. But at 44 days, for instance, the ratios of dry matter yields with P₁ and P₅ were N₁ : 4.0, N₂ : 11.0, N₃ : 17.0, N₄ : 20.8, and N₅ : 17.0.

Mitscherlich sought to answer some of the criticisms by his introduction of the damage factor. If this were assumed constant, like the effect factor, the ratio of yields with two concentrations of one nutrient should still not be affected by other environmental factors. But Mitscherlich did not claim constancy of the damage factor. It was an unspecified function of all the environmental variables, which certainly greatly reduced the attractive simplicity of his

hypothesis. Thus, to fit the data of a 5×5 interaction experiment (25 treatments) would, on his original theory, have needed three parameters only; it would now need six. Furthermore, adequate consideration has not been given to methods for fitting damage factors to observed interaction data.

As an alternative to Mitscherlich's formulation, the hypotheses suggested by Maskell (1925, 1928*a*, 1928*b*, 1929) (see also Brenchley, Maskell, and Warington 1927) — that the reciprocal of the yield could be expressed as the sum of elements each a function of the level of one external factor — may be tested. That is

$$\frac{1}{y} = F(N) + F'(P) + \dots,$$

where y is the yield, and N, P, \dots the amounts of the various nutrients supplied. This, by analogy with electrical theory, was called the "General Resistance Formula." A special form of the hypothesis (the "Special Resistance Formula"), in which the function was proportional to the reciprocal of the supply, was also studied; thus

$$\frac{1}{y} = \frac{a_n}{n + N} + \frac{a_p}{p + P} + \dots,$$

where y, N , and P have the meanings indicated above, n and p are the available amounts of the nutrients initially present in the substrate, and the other terms are constants. Balmukand (1928) developed statistical methods for testing these hypotheses, and found that they gave very satisfactory fits to nutrient interaction data in field trials and pot-culture experiments; so far as we are aware, however, no one has since analysed nutrient interaction data in this way.

Among the various sets of data in the present experiment showing significant first order interactions, one was taken at random for the purpose of testing Maskell's hypotheses; this was the shoot dry weight at 44 days, for the various combinations of nitrogen and phosphorus treatments. Both general and special hypotheses were tested, and the results are shown in the analysis of variance in Table 11.

The expectations from the general formula are in very satisfactory accord with the observations. The values of $F(N)$ and $F'(P)$ for the five levels of nitrogen and phosphorus supply used are:

Level of Supply	$F(N)$	$F'(P)$
1	0.00633	0.04266
2	0.00036	0.03051
3	-0.00057	0.00747
4	-0.00042	0.00405
5	0.00045	0.00265

when y, N , and P are all expressed in mg. Even in the case of the Special Resistance Formula, the deviations from expectation did not quite reach significance, although this hypothesis cannot cover the possibility of yield decreasing when

nutrient supply increases above a certain point — as has occurred in this lettuce experiment with nitrogen supply. In these data, the expression becomes

$$\frac{1}{y} = \frac{0.1470}{-31 + N} + \frac{0.1292}{1 + P} + 0.00194.$$

The negative value of n can hardly have any physical meaning, and may presumably be ascribed to the exigencies of fitting data in which the nutrient supply enters the supra-optimal region.

The degree of agreement attained suggests that this method of analysis, particularly the General Formula, deserves more attention than it has been accorded in the past by students of nutrient interaction.

Serious studies of nutrient interaction have generally led to conclusions similar in the main to those emerging in the present work. The work of Gregory with barley (Goodall and Gregory 1947), and that of Wittwer, Schroeder, and Albrecht (1947) with spinach likewise show the increase in magnitude of proportional responses to one nutrient with increasing supply of another, and up-

TABLE 11

ANALYSIS OF VARIANCE OF DATA FOR LOGARITHMS OF SHOOT DRY WEIGHT AT 44 DAYS, NITROGEN AND PHOSPHORUS COMBINATIONS, AFTER FITTING MASKELL'S FORMULAE

Source of Variation	Degrees of Freedom	Mean Square	<i>F</i>	<i>P</i>
General Formula	8	6.6909	71.714	<0.001
Residual	16	0.1203	1.289	>0.20
Special Formula	4	13.1040	140.450	<0.001
Residual	20	0.1518	1.627	0.05-0.10
Error (see Table 1)	64	0.0933		

ward displacement of the optimum amount of one as the supply of another is increased. These features of nutrient interaction results have often led to emphasis being placed on the concept of "nutrient balance." Various meanings have been ascribed to this phrase. Gregory (1937), for instance, regarded a balanced nutrient solution for barley as one in which the proportions of the nutrients were similar to those in barley grain. He pointed out that, for some of his interaction experiments, in a series of nutrient solutions in which the supply of two nutrients was increased in the same proportion, the growth and yield of the plants were linearly related to nutrient supply. This does not appear to be true for the present lettuce data. Indeed, it would be surprising if it were true in general. Increases in plant size generally carry with them changes in morphology, anatomy, and composition, and it is therefore reasonable to expect that the optimal ratios of nutrients will not be identical at different levels of nutrition.

In the case of most investigators who have referred to nutrient balance, no satisfactory definition is given; indeed, there seems to be an aura of loose

thinking around this concept. In general, the argument would appear to be that different nutrients have mutual optima—that for any given level x of a nutrient X there is a level y of nutrient Y giving maximum yield, and that if plants are supplied with x of X and y of Y then not only does an increase in the supply of Y reduce the yield but the same is also true of X . To put it geometrically, the surface connecting yield with supply of these two nutrients has a ridge, and from any point on the ridge only directions representing increases in the supply of *both* nutrients also represent increases in yield. The advocates of nutrient balance have in no case put forward a collection of data sufficient to enable such a relationship to be demonstrated rigorously, and the present lettuce data do not suggest a surface of that type.

VI. ACKNOWLEDGMENTS

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THE INTERACTION OF LIGHT AND TEMPERATURE IN DETERMINING THE GROWTH RATE OF SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM* L.)

By J. N. BLACK*

[Manuscript received March 15, 1955]

An experiment is described in which the growth of subterranean clover (*Trifolium subterraneum* L.) in the early vegetative stage was measured over 52 consecutive weekly periods. To eliminate possible trends of growth rates with age, plants of comparable morphological stage were used for each period. The variety Bacchus Marsh was grown in pot culture in the open at the Waite Agricultural Research Institute, Adelaide, South Australia.

Determination of dry weights (including those of the root fraction) and leaf areas enabled growth to be analysed in terms of relative growth rate, net assimilation rate, and leaf area ratio. Records of total light energy and temperature (daily mean, daily mean maximum, and daily mean minimum) were kept.

Statistical analysis by the method of simultaneous solution of multiple linear regressions demonstrated significant positive effects of light and maximum temperature and a significant negative effect of minimum temperature on net assimilation rate. It was shown that the value of the leaf area ratio at the final harvest was affected positively by the initial value, which may be taken as summing the pre-experimental environmental conditions, and by temperature (positively) and by light (negatively) during the experimental period. Relative growth rate was shown to be independent of temperature but significantly correlated with the amount of light received.

I. INTRODUCTION

The interaction of light and temperature in determining the rate of plant growth has been assessed for relatively few plant species. Gregory (1926), Goodall (1945), Watson (1947), and Blackman, Black, and Kemp (1955) have sought to relate variations in the relative growth rate, net assimilation rate, and leaf area ratio (or relative leaf growth rate) to fluctuations in total radiation and temperature, the plant species investigated being barley, tomatoes, potatoes and sugar-beet and wheat, and sunflowers respectively. The present investigation was undertaken to assess the influence of environmental factors on the growth of subterranean clover (*Trifolium subterraneum* L.), and forms part of a programme of research in progress at the Waite Agricultural Research Institute into the growth and development of this important pasture species.

The growth of a leguminous plant has not been studied in any of the previous investigations in which the effects of light and temperature have been independently determined. The special problems introduced by the presence of nitrogen-fixing bacteria in the root nodules were avoided as far as possible by

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inoculating the soil with an effective strain of *Rhizobium* and completing the experiments while the plants were still in the early vegetative phase. It was not thought possible to assess the separate effects of the environmental factors on the behaviour of the nitrogen-fixing bacteria and thus on the metabolism of the host plant, though regular examination of the nodules suggested that they were always well established and healthy. It must be borne in mind, therefore, that the effects of light and temperature on the growth of subterranean clover reported in this investigation may not in all cases be direct effects, but may contain elements of their effect upon the nodule bacteria.

In other ways, the use of subterranean clover as experimental material has much to commend it. The seed germinates regularly and quickly under optimal conditions. The species is self-pollinated, and a large number of easily recognizable strains have evolved, all of them breeding true for a number of characteristics, both morphological and physiological; as a result, the error introduced by variability of plant material is greatly reduced. The roots are strong and do not break up when washed out of the soil, and determination of leaf areas can readily be made, despite the small size of the early leaves.

II. EXPERIMENTAL METHODS

(a) *General*

In attempting to establish quantitative relationships between factors of the environment and the growth rate of plants, it is essential to separate fluctuations in growth rate caused by the changing environment from changes attributable to ontogenetic drifts. While it is never possible to eliminate entirely the effects of such drifts, the use of plants at a similar morphological stage for each determination of growth rate in the present experiment presupposed that growth rate was then influenced primarily by environmental conditions. Since the water and nutrient factors of the environment could be controlled, the growth rate measured over weekly periods was related to light energy and temperature, which, if they cannot be controlled, can at least be measured. Each determination of growth rate was based on two sampling occasions, both from plants sown at the same time, the first being taken when the plants had reached the second trifoliate leaf stage. Fifty-two consecutive measurements of growth rate over weekly periods were made, starting in October 1952, and finishing in October 1953.

(b) *Pot Culture Methods*

Standard 8-in. porous flower pots, with a basal drain hole, were used throughout the experiment; the whole inner surface was coated with bituminous paint in order to reduce loss of water. All the experimental pots were kept under natural open air conditions in a plunge bed of moist sawdust and were watered daily. Although very high shade temperatures, often exceeding 100°F, were encountered, no plants were ever observed to wilt.

The pots were filled to within an inch of the top with a sandy soil, and 1 g of superphosphate was added to each. In view of the rapid drying out of

the surface soil in summer and the difficulty of germinating subterranean clover in temperatures exceeding 90°F (32°C), it was decided to germinate all the seed at 18°C in an incubator for 24 hr, after which it was sown on the surface of the soil and covered with sand to the level of the top of the pots. All pots were inoculated with a liquid culture of *Rhizobium trifolii*, and after emergence the number of seedlings in each was reduced, usually in three stages, to six. The Bacchus Marsh strain of subterranean clover was used throughout, all seed being drawn from the same sample. Sowing dates were so adjusted that, each week, one batch of plants reached the standard initial stage of development, with the second trifoliate leaf beginning to expand. It was not always possible to judge this time interval correctly, but it had previously been established that over a considerable portion of the period of early vegetative growth, the rate of growth in the same environmental conditions remains constant. In accordance with the results of a simple uniformity trial conducted before the main experiment was designed, it was anticipated that 12 pots, each with six plants, would be required for both initial and final harvests and, in fact, each sowing consisted of 28 pots, four of which were subsequently rejected. Pots were allocated at random to the two sampling occasions.

(c) Sampling Procedure

Every Wednesday morning, 12 pots from one sowing were withdrawn for determination of "initial" weight; the following week, the other 12 were used for a "final" weight determination. Thus each week 24 pots were sampled, 12 for an initial and 12, from the previous sowing, for a final weight.

The plants were cut off at ground level and leaves separated from stems, petioles being included in the stem fraction. Leaves of one sample plant selected at random from each pot were set aside for determination of leaf area. Roots were recovered by washing, first through a sieve which retained all root material, and secondly in a dish where roots were separated from organic and other contaminating matter. All plant material was dried in an oven at 100°C for 24 hr before weighing.

Estimates of leaf area were made by spreading out the leaves of the sample plant on blueprint paper and exposing them to the sun. The areas of these leaf prints were subsequently determined with a planimeter and the total leaf area per pot was calculated from the area-weight ratio of the leaves of the sample plant and the known total leaf weight for each pot. Apart from the isolation of the leaves of one plant for blueprinting, no separate data were taken on the individual plants of each pot.

(d) Measurement of Light and Temperature

Blackman, Black, and Martin (1953) described an integrating recorder for the measurement of daylight designed for use in a similar experimental analysis of the growth of sunflower. An instrument using the identical circuit and components, differing only in the absence of a filter to eliminate infra-red radiation, was constructed and placed in the meteorological station of the Waite Institute.

Under the climatic conditions to which this instrument was exposed, there was a drift of sensitivity with aging, but regular calibrations were made and appropriate conversion factors calculated.

Mean temperatures for each period were obtained with a planimeter from the charts of a thermograph kept in a standard Stevenson screen. Mean daily maximum and minimum temperatures, which were used as an index of "day" and "night" temperatures respectively, were obtained by use of maximum and minimum thermometers.

The choice of an area for the experiment was limited to some extent by the need for proximity to the laboratory and to a water supply, and the plunge beds were eventually built at a distance of about 100 yd from the meteorological instruments. During the winter months, plants at the eastern end of the plunge beds came under the shade of trees in the early morning only, but care was taken to see that pots were removed from this end as the initial sampling occasion approached.

III. RESULTS

(a) *Techniques of Growth Analysis*

In a recent review, Watson (1952) has discussed the uses and limitations of the techniques of growth analysis, and a detailed elaboration of the methods will not be attempted here.

Net assimilation rate (Gregory 1917) was defined as the rate of increase in total plant weight per unit of assimilating material, i.e.

$$\text{Net assimilation rate} = \frac{1}{A} \times \frac{dW}{dt},$$

where W = plant weight, t = time, and A is a measure of the assimilating material. Between two sampling occasions (t_1 and t_2) the mean net assimilation rate can be calculated from the formula

$$\frac{W_2 - W_1}{A_2 - A_1} \times \frac{\log_e A_2 - \log_e A_1}{t_2 - t_1}.$$

At various times, different measures of "assimilating material" have been proposed; leaf area has been most generally used, but leaf weight (e.g. Goodall 1945) or leaf protein (e.g. Williams 1939) have been substituted. Watson (1952), in discussing the use of these various bases, has stressed the use of leaf area for the sake of uniformity, and it has accordingly been used in the present investigation.

Relative growth rate or efficiency index (Blackman 1919) was defined as the increase in plant weight per unit initial weight per day,

$$\frac{1}{W} \times \frac{dW}{dt},$$

and between two sampling occasions the mean value can be calculated from the formula

$$\frac{\log_e W_2 - \log_e W_1}{t_2 - t_1}.$$

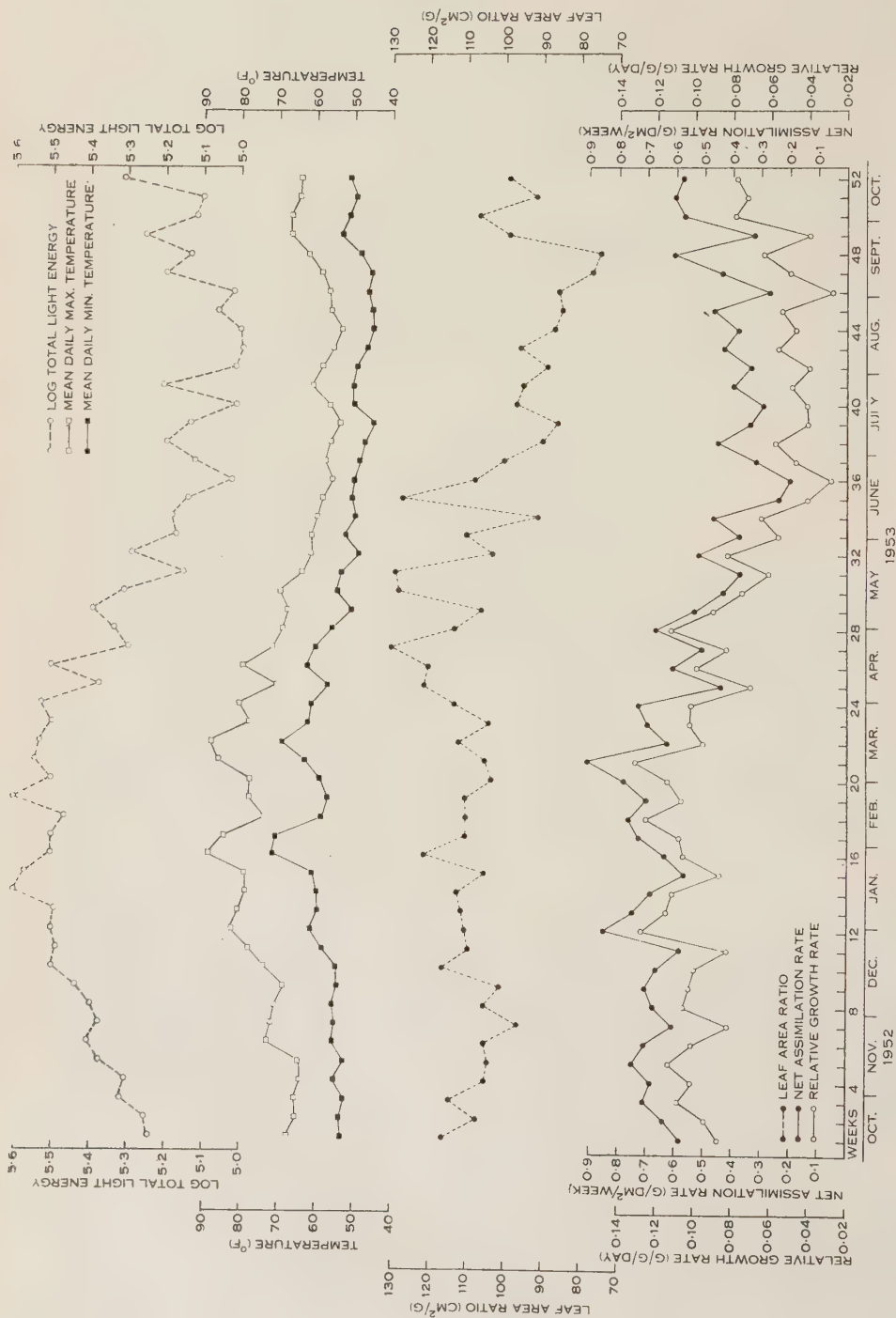


Fig. 1.—Seasonal trends in total light energy, mean daily maximum and minimum temperatures, net assimilation rate, leaf area ratio, and relative growth rate.

Briggs, Kidd, and West (1920) pointed out that relative growth rate is the product of net assimilation rate and the ratio of leaf area to plant weight, or the leaf area ratio, and these interrelationships have been further examined by Blackman and Wilson (1951*b*). It is apparent that environmental influences on relative growth rate must operate through net assimilation rate or leaf area ratio, or both.

Williams (1946) has stressed the fact that the integrated form of the net assimilation rate equation is approximate, as it assumes a linear relationship between plant weight and leaf area during the experimental period. However,

TABLE 1

REGRESSION CONSTANTS RELATING NET ASSIMILATION RATE, LEAF AREA RATIO, AND RELATIVE GROWTH RATE TO ENVIRONMENTAL FACTORS

$$(y = a + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_6x_6)^\dagger$$

Growth Index (<i>y</i>)	Regression Constants						
	<i>a</i>	<i>b</i> ₁	<i>b</i> ₂	<i>b</i> ₃	<i>b</i> ₄	<i>b</i> ₅	<i>b</i> ₆
Net assimilation rate (g/dm ² /week)	-1.803	0.407* ±0.183		0.0155* ±0.0062	-0.0158* ±0.0071		
or	-1.770	0.397** ±0.142				0.0155* ±0.0061	
Leaf area ratio (cm ² /g)	165.10	-35.885* ±15.109	1.262*** ±0.327				0.543*** ±0.107
Relative growth rate (g/g/day)	-0.602	0.129*** ±0.012					

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

*** Significant at $P < 0.001$.

† x_1 = logarithm of total light energy; x_2 = mean temperature; x_3 = mean max. temperature; x_4 = mean min. temperature; x_5 = mean daily range of temperature; x_6 = initial leaf area ratio.

since the time intervals in the present investigation were short (7 days), serious departures from linearity would be unlikely and the net assimilation rate formula quoted above could be used with confidence.

It was accordingly possible to calculate relative growth rate, net assimilation rate, and leaf area ratio for each of the 52 consecutive weeks of the experiment. In Figure 1, the data are plotted graphically to show the seasonal trends in both environmental conditions and the measures of growth rate employed. The logarithm of the total light energy received is used since Blackman and Wilson (1951*b*) had shown that, for a number of species (including subterranean clover) the relationship between light intensity and both net assimila-

tion rate and leaf area ratio is logarithmic over a wide range of shading treatments.

The seasonal trends of relative growth rate and net assimilation rate are seen (Fig. 1) to correspond in general to the trends in light and temperature, but it is clearly impossible from this method of presentation to determine the separate effects of the two environmental factors on growth rates. The data were accordingly submitted to statistical analysis and the results will be considered separately for each measure of growth.

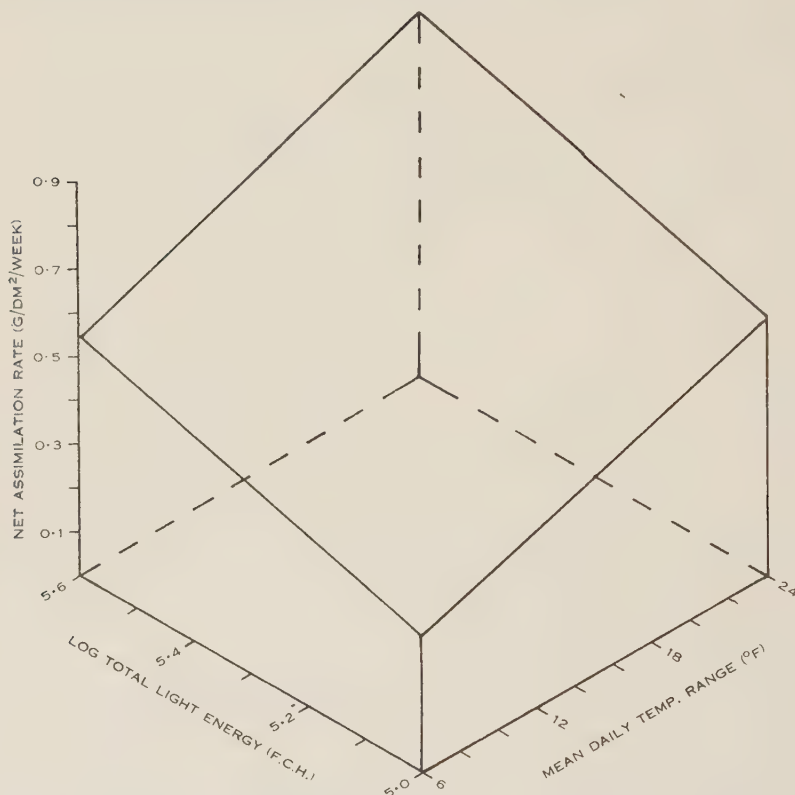


Fig. 2.—Interaction of total light energy (f.c.h.) and mean daily temperature range in determining net assimilation rate.

(b) *The Interaction of Light and Temperature in Determining Net Assimilation Rate*

The analysis of the net assimilation rate data by means of a multiple linear regression on the logarithm of the total light energy and temperature showed a significant positive effect of light ($P < 0.05$), a significant positive effect of mean maximum temperature ($P < 0.05$), and a significant negative effect of mean minimum temperature ($P < 0.05$). The regression coefficients of the two temperature indices were almost equal in absolute value (a test of significance of the difference gave a probability of 90 per cent.). It was therefore possible

without loss of accuracy to replace the mean maximum and mean minimum temperatures in the regression equation by a single index, the mean daily range of temperature. It also followed that the mean daily temperature has no effect on net assimilation rate when the effect of light is eliminated. The effect of mean daily range of temperature is positive and significant ($P < 0.05$). The appropriate regression coefficients are given in Table 1. The interaction of light and temperature in determining net assimilation rate is shown in Figure 2.

An alternative method of illustrating these data is based on the departure of the values of net assimilation rate calculated from the regression equation from the observed values: if the calculated values are plotted against the observed values, the fit of the calculated regression will be paralleled in the extent to which the data depart from the strict linear relationship. This procedure is illustrated in Figure 3.

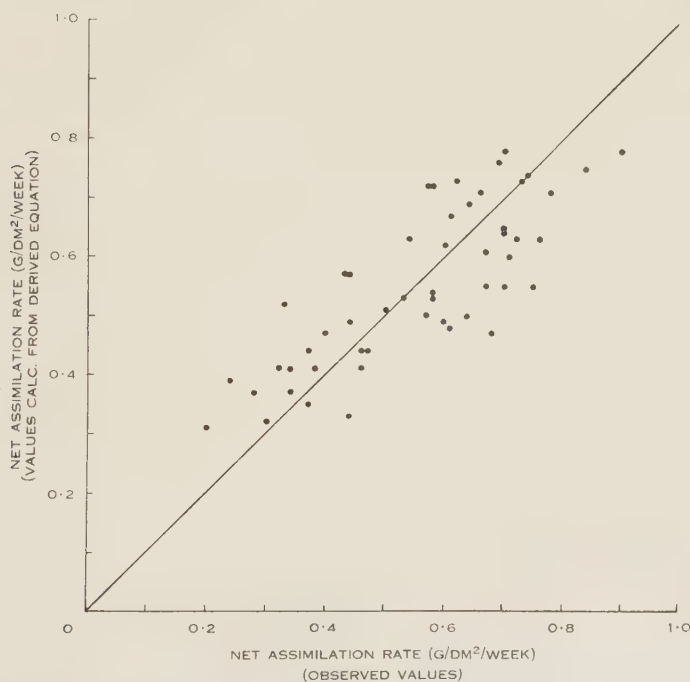


Fig. 3.—Agreement between observed and calculated values of net assimilation rate.

At this point it is necessary to observe that the use of regression analysis to determine the separate effects of several predicting variables may lead to some instability in the regression coefficients where the predicting variables are themselves intercorrelated. By continuing the experiment throughout the whole year it was hoped to obtain the greatest possible number of combinations of light and temperature conditions, but the limitations of the form of analysis performed must not be overlooked.

(c) *The Interaction of Light and Temperature in Determining Leaf Area Ratio*

In the analysis of net assimilation rate it was assumed that the measured rate could be attributed to the current environment and that a mean net assimilation rate could reasonably be related to mean — or total — values of light and temperature. This assumption cannot be made in the analysis of leaf area ratio, the value for which at any one time must to a certain extent reflect the whole

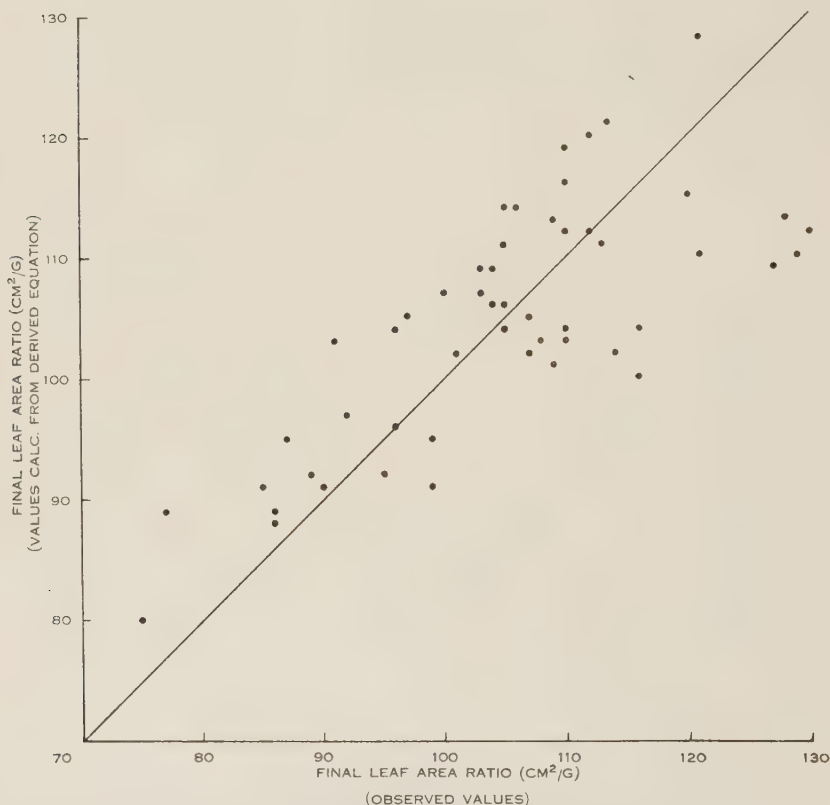


Fig. 4.—Agreement between observed and calculated values of leaf area ratio.

previous history of the plant; in particular, the use of the mean value — in this case the mean of the leaf area ratios at the initial harvest and the final harvest 7 days later — would be inappropriate in view of the evidence presented by Blackman and Wilson (1951*b*) that the response of this species to imposed shading is markedly slower than for other species investigated. Furthermore, Blackman, Black, and Kemp (1955) have shown that, for sunflower, there is a seasonal effect on leaf area ratio which is best interpreted as a residual effect of the previous environment. If the value of the leaf area ratio at the final harvest is taken as the dependent variable, and the initial value added to the environmental factors as a further independent variable, it should be possible

to determine whether in fact the conditions prior to the first harvest — as reflected in the initial value of the leaf area ratio — have any significant effect on final values, and also to what extent the final value is affected by the environmental conditions between harvest occasions when the initial value is held constant.

The analysis performed in this way demonstrated a significant ($P < 0.001$) and positive effect of the initial value in determining the final value of leaf area ratio. The effect of total light energy was negative and was significant ($P < 0.05$). Both mean maximum and mean minimum temperatures significantly affected final leaf area ratio, and as a result of the close correlation between them either could be used alone without affecting the goodness of fit of the regression; it was therefore decided to use the mean temperature. As an effect of temperature range had been noted in the analysis of net assimilation rate this too was tested, but no significant effect was demonstrated.

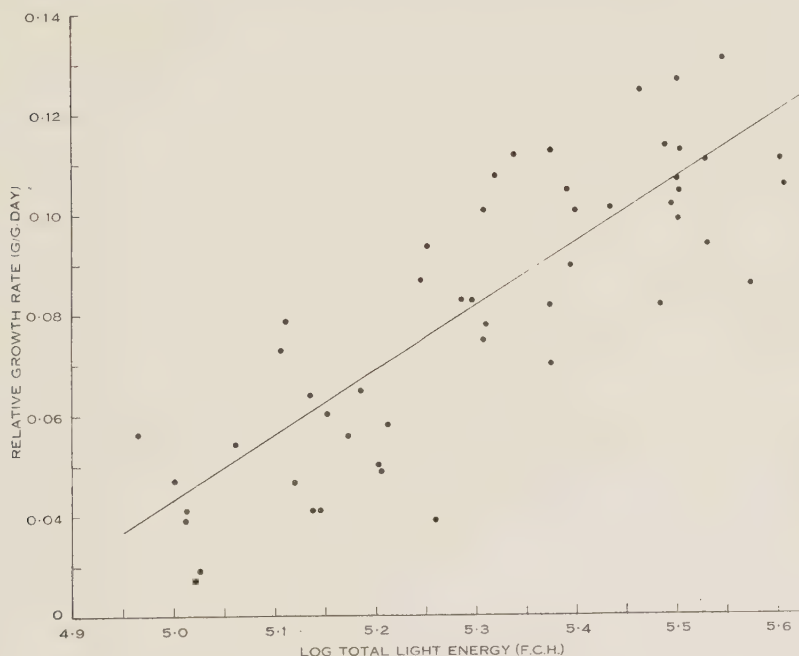


Fig. 5.—Effect of varying total light energy on relative growth rate.

The regression coefficients relating final leaf area ratio to initial value, to the total light energy, and to mean temperature are given in Table 1, and the correspondence between final values calculated from the regression equation and the observed values are illustrated in Figure 4.

(d) The Interaction of Light and Temperature in Determining the Relative Growth Rate

It is clear from Figure 1 that relative growth rate follows very closely the seasonal trend of net assimilation rate, and that since variation in leaf area

ratio had been minimized by the use of plants of comparable morphological condition, the factors affecting net assimilation rate are likely to be those determining relative growth rate. Analysis in terms of the logarithm of the total light energy and the various temperature indices demonstrated, however, a positive and significant ($P < 0.001$) effect of light but no effect of temperature; the appropriate regression coefficients are to be found in Table 1. The relationship between relative growth rate and total light energy is shown in Figure 5.

IV. DISCUSSION

It is of considerable interest to compare the results obtained from the present investigation with others in which a quantitative relationship between growth and environmental factors has been attempted. These are fewer than might have been expected in view of the importance of climate in agricultural crop production, and only seven species have been examined in a comparable manner: maize (an analysis of Kreuzer's data by Briggs, Kidd, and West 1920), barley (Gregory 1926), tomatoes (Goodall 1945), wheat, sugar beet, and potatoes (Watson 1947), and sunflower (Blackman, Black, and Kemp 1955). For barley, tomato, sunflower, and subterranean clover there is a significant and positive effect of light on net assimilation rate, and for no species has a significant negative effect been demonstrated. This is in accord with the results of Blackman and Wilson (1951*a*), who demonstrated a linear relationship between net assimilation rate and the logarithm of light intensity relative to full daylight for 10 species. Unpublished data of Blackman and Black confirmed this relationship and extended its application to a number of graminaceous and leguminous species of agricultural importance.

The influence of temperature on the net assimilation rate of barley (Gregory 1926) and subterranean clover is similar, in that there is a positive effect of day (or maximum) temperature and a negative effect of night (or minimum) temperature; in both cases these effects, though opposite in sign, are equivalent in magnitude. Gregory (1926), in discussing the barley data, suggested that high night temperatures increase respiration rate, thus reducing net assimilation rate, and that high day temperatures increase photosynthesis, thus increasing net assimilation rate, and it is probable that the same explanation holds for subterranean clover. The opposite effects of day and night temperatures suggest that analysis in terms of mean temperature is unlikely to yield a significant relationship with net assimilation rate; in fact mean temperature is the least satisfactory index, the mean daily range being a far preferable single value index. Clearly the wider the range of temperature the greater the net assimilation rate. However, while the range has the numerical advantage of giving a single value determinant, it is possible that, if Gregory's explanation is correct, more accurate data would demonstrate that the positive effect of day temperature and the negative effect of night temperature are independent; in these circumstances the use of the diurnal range would be inappropriate.

The importance of the range of temperature has been stressed by Went (e.g. 1944), and the concept of thermoperiodicity has been developed to

cover the results of the series of experiments in which the growth of a number of plant species has been investigated in controlled conditions. Increases in stem height and not changes in dry weight have been the usual index of growth and the experiments have demonstrated temperature relationships similar to those reported here for net assimilation rate. It would appear that temperature range may be an environmental factor of greater importance than has been appreciated in the past. These opposing effects of day and night temperature were not, however, shown for maize, tomato, potato, wheat, sugar-beet, or sunflower.

The negative effect of light and the positive effect of mean temperature on final leaf area ratio, initial values being held constant, is in agreement with the findings of Blackman, Black, and Kemp (1955), the only comparable study in which leaf area ratio was examined. It is of interest to note that Blackman and Wilson (1951*b*) found that marked changes in the light environment of subterranean clover (shading to 0.50, 0.24, and 0.12 daylight) brought about only a small increase in leaf area ratio, but that when the plants were sampled after a further 9 days at the imposed shading conditions, a more marked increase in leaf area ratio was observed, this increase being greatest at low levels of light intensity, but still of only a small order at 0.50 daylight. It would seem, therefore, that the rate at which the leaf area ratio of subterranean clover becomes adapted to changes in the light environment is slow. It is considered that this lack of plasticity of response to changing environmental conditions is largely responsible for the importance of initial values in determining final values of leaf area ratio. While it was possible to put forward a tentative physiological explanation of the effect of temperature on net assimilation rate, it is not possible to do so for leaf area ratio, which, being a ratio rather than a rate, is much less capable of direct physiological explanation.

Both Gregory (1926) and Blackman and Wilson (1951*b*) have stressed that there exists in the species studied by them a mechanism whereby the growth rate is maintained approximately constant despite changes in radiation or light intensity; the contrasting effects of a decreasing net assimilation rate and increasing leaf area ratio with shading combine to protect the plant over a range of light conditions. While in the present experiment there are contrasting effects of light on net assimilation rate and leaf area ratio, analysis of relative growth rate shows that subterranean clover is not protected against changes in the light environment since the negative effect on leaf area ratio is more than offset by the positive effect on net assimilation rate. It is important to note that in the experiments of Blackman and Wilson the leaf area ratio used is the mean of initial and final values, whereas for the present analysis the final value was considered more appropriate; as a result it is not possible to analyse with the same precision the relative contributions of net assimilation rate and leaf area ratio to relative growth rate, and since there is an effect of pre-experimental environment on leaf area ratio, there must also be a similar effect on relative growth rate. However, such a pre-conditioning effect is unlikely to affect net assimilation rate (Blackman and Wilson 1954) and it is clear from Figure 1 that fluctuations in relative growth rate are attributable more to varia-

tions in net assimilation rate than to leaf area ratio. Blackman, Black, and Kemp (1955) were unable to demonstrate a seasonal effect on either net assimilation rate or relative growth rate although both that investigation and the present one have shown that leaf area ratio cannot adequately be analysed without taking into account the influence of the pre-experimental environment. It is accordingly clear that further detailed experimentation on the influence of residual environmental effects on net assimilation rate and relative growth rate, as well as on leaf area ratio, is now required before the influence of environmental factors on growth rate can be established with greater precision.

From the interaction of net assimilation rate and leaf area ratio Blackman and Wilson (1951*b*) calculated by extrapolation that the light intensity at which the growth rate of subterranean clover would be maximal was 1.87 Oxford summer daylight. The mean summer light energy per week received at Oxford (latitude 52°N) during 1950 and 1951 (May-August) was 214,460 f.c.h.; 1.87 of this is 401,040 f.c.h., a value which was exceeded only twice in Adelaide (latitude 35°S), where the most light energy received in any one 7-day experimental period was 405,710 f.c.h. Even at high values, no deviation from the logarithmic relationship between relative growth rate and total light energy could be demonstrated, and it is possible that the light intensity for maximal growth rate is even higher than the figure quoted.

On the basis of the data presented here, it would appear that the rate of growth of subterranean clover is independent of temperature, at least within the range of temperatures studied. There is presumably a minimum temperature below which growth does not proceed, but there is no evidence that temperatures of this order were experienced during the course of this experiment, in which the lowest weekly mean temperature was 47.6°F. There can be little doubt that growth is limited by low temperatures in some parts of southern Australia, and C. M. Donald (private communication) has suggested that they are a constant feature of the winter environment of Canberra, A.C.T. (mean temperature of coldest month, July, is 42°F). On the other hand, there is no evidence that growth is limited by high temperatures. It is clear that within the range of temperatures at Adelaide (weekly means during the course of the experiment from 46.7 to 77.3°F), the rate of growth of this species in the early vegetative stage is determined only by the amount of light energy received.

V. ACKNOWLEDGMENTS

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THE EFFECT OF LIGHT ON ZINC DEFICIENCY IN SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM* L.)

By P. G. OZANNE*

[Manuscript received March 3, 1955]

Summary

The effects of light on zinc response were investigated by growing subterranean clover under daylight of various intensities. Plants were grown in limed Muchea sand and the zinc response was measured as the difference in dry weight between plants with and without added zinc. The light intensities are given as the mean daily maximum intensities.

Zinc responses increased as light intensities increased from 200 f.c. up to 3000-4000 f.c. Further increases up to 11,000 f.c. caused a decrease in zinc response. Maximum zinc responses occurred at light intensities which were near saturation levels for photosynthesis.

Increased light intensities gave decreased concentration of zinc in the green leaves of the plants.

Plants grown under an 11½-hr day showed a much greater zinc response than plants under a 7½-hr day. The long day plants took up more zinc than the short day plants but retained a relatively larger proportion of zinc in the roots.

The relation between light intensity and zinc response is discussed.

I. INTRODUCTION

In the south-west of Western Australia, plant responses to zinc-containing fertilizers have been frequently reported (e.g. Teakle 1942; Dunne and Elliot 1950). Although these responses to zinc are widespread, they are by no means consistent, and in the same field, may vary from month to month and year to year. Deficiency symptoms are usually most severe in the winter months under low light intensity and short day conditions, the affected plants recovering towards summer (Dunne, Smith, and Cariss 1949; Rossiter 1952).

Experiments reported by Hoagland (1944) and Trumble and Ferris (1946) show that light intensity and daily light duration can influence zinc deficiency. However, in contrast to the field observations mentioned above, Skoog (1940) and Millikan (1953) reported that zinc deficiency symptoms were more severe under brighter light. These workers grew most of their experimental plants in water culture. Under such conditions the effects which light may have on the ability of plants to absorb zinc from the relatively unavailable sources in the soil cannot be shown.

The experiments described in this paper were designed to examine the effects of a wide range of light intensities on plants growing in a zinc deficient soil.

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II. EXPERIMENTAL METHODS

Subterranean clover plants (*Trifolium subterraneum* L.) were grown from commercial seed inoculated with an effective strain of *Rhizobium*. The plants were grown in white, glazed porcelain pots each containing 2 kg of Muchea sand which field trials by Rossiter (1951a) have shown to be zinc deficient. The topsoil used is a grey sand consisting mainly of silica with about 3 per cent. organic matter; it contains approximately 0.7 p.p.m. of zinc. The sand is too acid (pH 5.2) for good nodulation by subterranean clover and in all experiments 2.8 g CaCO_3 were mixed in dry to each pot. This raised the soil pH to 6.5 and led to good nodulation.

In addition to the 2.8 g of CaCO_3 , the following basal dressing (mg per pot) was applied at sowing: K_2SO_4 , 210; $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$, 51; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 70; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 15; $\text{FeSO}_4 \cdot 5\text{H}_2\text{O}$, 15; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 15; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 1.5; H_3BO_3 , 6. To prevent iron deficiency symptoms, 15 mg of tartaric acid were added to the iron solution. This basal dressing was prepared from A.R. chemicals.

Pots were maintained at 40 per cent. of the water holding capacity of the soil by additions of water distilled from a "Pyrex" all-glass apparatus.

In each trial half the plants were grown as low zinc (Zn_0) treatments with no added zinc, and to the remainder, 35 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ per pot were added to give a set of high zinc (Zn_1) or complete nutrient control treatments.

The various light intensity treatments used over the range of 5000-12,000 f.c. represent full daylight in the glasshouse at different seasons between mid-winter and midsummer. Light intensities between 200 and 4000 f.c. were obtained by shading the plants with white cloth screens of the required thickness. These screens were open at the sides to allow free air circulation (see Plate 1, Fig. 1). The light intensities were read at plant level on an E.E.L. light-meter.* They are expressed as the mean of the maximum daily readings taken during each experiment.

The mean day lengths reported were measured from sunrise to sunset as the light intensities at other times were considered to be too low to affect the vegetative growth of subterranean clover.

In every case, a factorial experimental design was used.

The tops and roots of all plants were harvested separately 42 days after germination and while still in the vegetative stage. At harvest, the roots were washed from the soil with tap water, then washed in dilute acetic acid, washed in distilled water, and finally dried in an oven at 85°C. The tops were hand separated into green leaves, dead leaves, and stems plus petioles, then oven dried at 85°C.

(a) Experiments 1-3

Experiment 1 germinated on January 1, 1952. Mean day length during the trial was 13 hr 50 min. At sowing, 140 mg of NaNO_3 plus an equivalent amount of HNO_3 were applied to each pot. Two light intensities were used—340 and

* Made by Evans Electroselenium Limited, Harlow, Essex.

750 f.c. Two strains of clover were grown—Dwalganup and Bacchus Marsh. Mean daily maximum and minimum temperatures were 30.0 and 18.9°C.

Experiment 2 germinated on February 26, 1952. Mean day length was 13 hr 7 min. At sowing, 35 mg of NaNO_3 plus an equivalent amount of HNO_3 were applied to each pot. Two light intensities were used—220 and 600 f.c. Dwalganup and Bacchus Marsh strains of subterranean clover were sown. Each treatment was replicated four times. Mean daily maximum and minimum temperatures were 30.0 and 18.9°C.

Experiment 3 germinated on April 7, 1952. Mean day length was 10 hr 57 min. No nitrogen was applied. Dwalganup strain only was sown. Light intensities used were 1050 and 7600 f.c. Three replications were sown. Mean daily maximum and minimum temperatures were 31.2 and 14.5°C.

(b) Experiment 4

Seed germinated on March 3, 1953. At sowing, NH_4NO_3 (350 mg per pot) was applied to half the pots. Three light intensities were used—"low" light at 750 f.c., "medium" light at 1400 f.c., and "high" light at 8000 f.c. Half the plants were grown under "long" day conditions of 11½ hr mean day length. The "short" day plants were covered daily at 2.0 p.m. with large light-tight black cardboard boxes and only received a mean of 7½ hr daylight.

The quantity of light received daily under the different treatments was found by recording the light intensity at short intervals throughout the day and plotting these values against time. By measuring the area under the curve so obtained, the mean quantity of light received daily under the various light treatments was found.

Dwalganup strain only was sown. Three replications were used. To ensure an even air temperature under all light intensities, the treatments were laid out in one glasshouse through which air was passed at a rate of 1 ft/sec. The air temperature, as read on a shaded thermometer, or the soil temperature did not vary to any extent from treatment to treatment. The mean daily maximum and minimum air temperatures were 30.7 and 17.2°C. The mean daily maximum and minimum relative humidity values were 89 and 50 per cent.

Zinc content of tops and roots was estimated using the wet digestion method described by Piper (1944) and a modification of the photometric method described by Cowling and Miller (1941).

Eight other trials, details of which will be reported elsewhere, were carried out using the same technique as described above. The mean daily maximum light intensities during these trials were recorded, and also the zinc responses for those fertilizer treatments used in experiments 1-4.

III. RESULTS

(a) Experiments 1-3

In these trials, zinc deficiency symptoms as described by Rossiter (1951b) and Millikan (1953) appeared first within 16 days of germination on the low zinc treatments. Symptoms were about 3 days later in appearing on plants

under the low light treatments of experiments 1 and 2. The mean yields from the treatments in experiments 1-3 are shown in Table 1. As no strain by zinc interaction was shown in experiments 1 and 2 the yields of the two strains are combined. The high plant weights of experiment 1 are probably due to the long day conditions and the relatively high level of applied nitrogen.

TABLE 1
EFFECT OF LIGHT INTENSITY ON THE ZINC RESPONSE OF SUBTERRANEAN CLOVER

Expt.	Light Intensity (f.c.)	Mean Dry Wt. Yield of Tops (g)		Zinc Response ($Zn_1 - Zn_0$)	L.S.D. of Two Means		Zinc \times Light Interaction
		High Zinc (Zn_1)	Low Zinc (Zn_0)		$P = 0.05$	$P = 0.01$	
1	340	0.765	0.566	0.199	—	—	Positive
	750	1.981	1.351	0.630	—	—	
2	220	0.259	0.222	0.037	0.059	0.081	Positive**
	600	0.808	0.647	0.161			
3	1050	0.875	0.648	0.227	0.121	0.183	Negative
	7600	0.662	0.554	0.108			

** Significant at $P = 0.01$ level.

Increasing the light intensity from 340 to 750 f.c. in experiment 1 gave markedly increased growth and also greatly increased zinc response as measured by the difference in yield between the low and high zinc treatments. In experiment 2, these light effects were confirmed as both plant growth and zinc response were much greater under a light intensity of 600 than 220 f.c. However, these two trials were carried out at relatively low light intensities.

During experiment 3 the higher light value used was the full autumn sunshine received through the roof of the glasshouse. In contrast to the effects in experiments 1 and 2, increasing the light intensity in experiment 3 from 1050 to 7600 f.c. gave no increase in plant weight and the zinc response was decreased.

A marked effect of light intensity on plant form was observed in these trials. The plants grown under a mean daily maximum of only 220 or 340 f.c. were tall and spindly in appearance; plants under 1050 f.c. were large with broad leaves; while the plants under sunlight at 7600 f.c. were dense and compact with short petioles and small leaves.

TABLE 2
EFFECT OF LIGHT INTENSITY AND DAY LENGTH ON THE ZINC RESPONSE OF SUBTERRANEAN CLOVER (EXPT. 4)

Day Length	Light Treatment	Light Intensity (f.c.)	Light Quantity (f.c.h.)	Mean Dry Wt. Yield of Tops (g)		Zinc Response (Zn_1-Zn_0)	L.S.D. of Two Means		Zinc \times Light Interactions
				High Zinc (Zn_1)	Low Zinc (Zn_0)		$P = 0.05$	$P = 0.01$	
Short day (7½ hr)	Low	750	3,700	0.354	0.252	0.102	0.093	0.126	Low <i>v.</i> Medium —Positive** Medium <i>v.</i> High —Negative*
	Medium	1400	7,100	0.740	0.429	0.311			
	High	8000	43,900	0.500	0.341	0.159			
Long day (11¼ hr)	Low	750	5,000	0.875	0.460	0.415	0.230	0.314	Low <i>v.</i> Medium —Positive*** Medium <i>v.</i> High —Negative* Short day <i>v.</i> Long day—Positive***
	Medium	1400	9,600	1.803	0.664	1.139			
	High	8000	61,300	1.691	0.979	0.712			

* Significant at $P = 0.05$ level.

** Significant at $P = 0.01$ level.

*** Significant at $P = 0.001$ level.

(b) Experiment 4

To clarify the apparently contradictory light effects of experiments 1 and 2 as compared with 3, experiment 4 was carried out using one relatively low light intensity, one intensity between those of experiment 3, and one bright sunshine intensity. The yields of this trial and the zinc responses obtained are shown in Table 2. As there was no significant interaction between applied nitrogen and zinc responses at different light intensities or at different day lengths, the yields of the two nitrogen treatments were combined.

Zinc deficiency symptoms occurred in experiment 4 as in the earlier trials, but did not appear on the low light short day treatments until day 24. However, by day 35, symptoms were showing on at least 40 per cent. of the leaves of all low zinc plants.

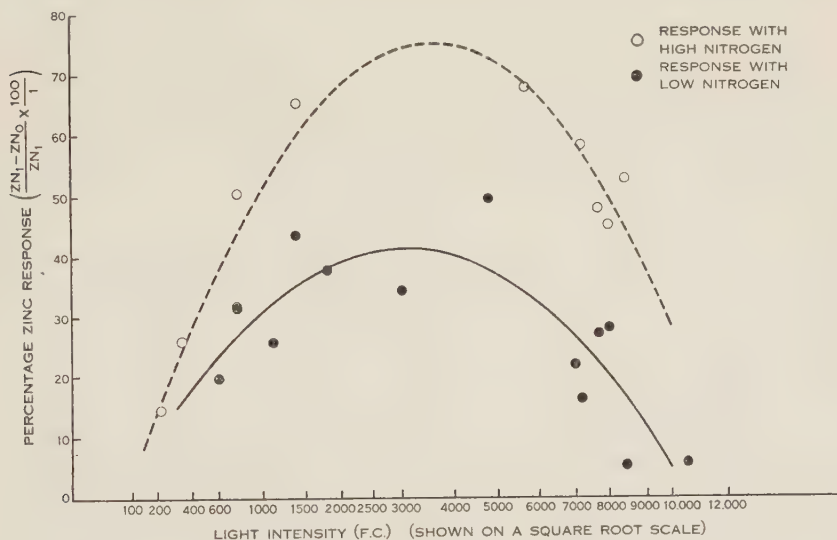


Fig. 1.—Zinc responses of subterranean clover plotted against light intensity. ○ Responses of plants supplied with ample combined nitrogen. The broken curve has been fitted to these points, and the multiple correlation coefficient (r) for these values is 0.94. ● Responses of plants relying on nitrogen from symbiotic nitrogen fixation in the root nodules. The solid curve has been fitted to these points, and $r = 0.82$ for these values.

(i) *Light Intensity*.—In experiment 4 the increase in light intensity from 750 to 1400 f.c. gave rise to a large increase in plant weight and also in response to applied zinc. However, the increase in light from 1400 to 8000 f.c. intensity failed to increase the dry weight production of the high zinc plants, and gave a decreased zinc response.

The trend of dry weight of tops between medium and high light in long day-low zinc plants differed from that of the other three treatments. However, as in all other trials, under high light intensity the yield of the low zinc plants was closer to that of the high zinc plants, and the severity of zinc deficiency symptoms was greatly reduced.

The yields from experiment 4, taken in conjunction with those from experiments 1-3, show an increase with increased light intensity up to 1400 f.c. of daylight. Near this intensity light seems to reach the saturating level for photosynthesis, and increasing the light intensity above 3500 f.c. did not give increased plant weight.

As in experiments 1-3, the zinc responses obtained in experiment 4 suggest that, up to a certain level, increasing light intensity leads to increasing plant responses to zinc. In an attempt to find the level of light giving the maximum zinc response with subterranean clover, the experimental data from experiments 1-4 and the other eight trials referred to above were used. The zinc responses obtained in these trials are plotted against the mean daily light intensity in Figure 1.

TABLE 3
EFFECT OF LIGHT ON ZINC CONTENTS AND ROOT WEIGHT RATIOS (EXPT. 4)

Day Length	Light Intensity	Low Zinc Plants				Root Wt. Ratio* (%)
		Zinc in Green Leaves (p.p.m.)	Zinc in Roots (p.p.m.)	Total Zinc in All Leaves (μ g)	Total Zinc in Roots (μ g)	
Short day (7½ hr)	Low	30.7	187	3.5	8.8	15.9
	Medium	25.7	151	6.8	15.6	19.4
	High	24.2	174	5.3	20.4	26.0
Long day (11¾ hr)	Low	26.7	145	5.6	18.0	19.2
	Medium	21.4	147	8.1	26.0	21.2
	High	20.9	127	11.6	40.0	27.1
		High Zinc Plants				
Short day (7½ hr)	Low	130	450	26	25	13.5
	Medium	74	278	32	44	17.8
	High	82	297	25	46	23.4
Long day (11¾ hr)	Low	73	312	32	55	16.8
	Medium	54	246	51	116	20.9
	High	56	218	50	143	28.1

* Root wt./total wt. \times 100.

For ease of comparison the zinc responses are graphed on a relative basis as the "percentage zinc response," or the difference in yields between high and low zinc treatments expressed as a percentage of the high zinc yield. As the responses to zinc are greater in plants with a high nitrogen supply (Ozanne 1955), these treatments are shown separately.

From Figure 1 it may be seen that the maximum response to zinc was given by the plants at about 3500 f.c. light intensity, and this peak was not moved to any extent by increasing the nitrogen status of the plants.

In experiment 4, as in the earlier trials, the increase in intensity of light had a progressive effect on the appearance of the plants which became more dense and compact with increasing light intensity as shown in Plate 1, Figure 2.

(ii) *Day Length and Quantity of Light*.—The plant yields under the various day length and light energy treatments are shown in Table 2. A large decrease in plant weight was obtained by reducing the daily duration of photosynthesis from 11½ to 7½ hr. No increase in light intensity was able to compensate for the shorter day length. Although the medium light and high light treatments under short day received 7000 and 43,000 f.c.h. of light respectively, the plants were still smaller and their dry weight less than those under low light, long day conditions receiving only 5000 f.c.h. of light.

In spite of their smaller size, the short day plants responded in growth form to the different levels of light intensity in a similar way to the long day plants. But the zinc responses obtained at the shorter day length were smaller on both an absolute and relative basis than the response under the longer day conditions.

(iii) *Zinc Contents*.—The living green leaves are the principal light receptors of the plant and most of the photosynthetic processes are carried out within them. For this reason, the zinc content of the green leaves was determined separately from the other plant parts. In the case of the low zinc treatments, this separate analysis was carried out to find the minimum zinc concentration required by the leaves under the various light treatments.

In general, increased light led to greater total zinc uptake. However, growth increases in response to more light tended to produce a dilution effect on the absorbed zinc, i.e. greater production of dry matter than uptake of zinc. The zinc analyses of plant material from experiment 4 are given in Table 3.

The zinc concentration in green leaves of both high and low zinc plants decreased significantly between low and medium light treatments, though differences between medium and high light are probably not significant. The decreases in zinc content with increased light intensity are the more striking since the level of zinc concentration in roots is still high at higher light intensities. At both levels of zinc, the short day plants give a similar pattern of analyses to the long day plants, but zinc concentrations are higher, and total uptakes lower, under the short day.

IV. DISCUSSION

The experimental evidence presented here indicates that the zinc requirement of subterranean clover is markedly affected by light intensity. Increasing zinc responses were obtained as light intensities were increased up to a daily maximum of about 3000-4000 f.c. Further increases in light intensity between 4000 and 11,000 f.c. gave corresponding decreases in zinc response. The latter

range of light values was obtained by using light intensities received between midwinter and midsummer. Because of this, plants grown under light values of 5000-7000 f.c. received an appreciably shorter day length than plants grown under light of 8000-11,000 f.c. intensity. However, in experiment 4, it was found that the longer day length gave the greater zinc response. Hence the decrease in zinc response found over the range of 4000-11,000 f.c. is unlikely to have been caused by the increasing day length.

Strong solar radiation falling on the growing plant has many complex effects, e.g. increase in leaf blade temperature, increase in respiration rate, and increase in transpiration rate with a possible consequent decrease in leaf turgor and partial closure of the stomates. Hence the decreased zinc response at high light intensity probably has no single explanation. It is worth noting in experiment 4, however, that the concentration of zinc present in green leaves under full sunlight was substantially the same as that found under thin shade, even though the plants under thin shade suffered more severely from zinc deficiency. It appears then that the leaves growing under high light intensity were able to utilize the zinc present in them more efficiently than leaves growing under the medium light intensity.

Light showed some effect on the absorption and distribution of zinc. The long day plants took up more zinc than did the plants under short day, but they also retained a relatively larger amount of the absorbed zinc in the roots. A similar effect of long day on zinc distribution within the plant was reported by Ferres (1951), and is partly due to the increased root weight ratio under long day conditions.

The high concentration and high total zinc content of the roots relative to the leaves is very striking, especially in the treatments which gave rise to acute zinc deficiency. Work reported elsewhere (Ozanne 1955) indicates that much of the zinc found in roots is held in complexes and is not readily mobile. For this reason the total zinc content of the roots relative to the leaves is affected to a considerable extent by the root weight to total plant weight ratio. This ratio was increased by every increase in total light energy received, irrespective of day length or light intensity.

Reports from California (e.g. Hoagland 1944) state that corn, tomato plants, and citrus trees show zinc deficiency most frequently under summer conditions of high light intensity. By contrast, observations made in southern Australia indicate that subterranean clover, flax, and oats suffer most severely from zinc deficiency during the cloudy winter weather of lower light intensities. In so far as subterranean clover is concerned, these latter observations are supported by the experiments described here, in which the greatest responses to zinc were obtained under light values of the same order as those received in midwinter.

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EXPLANATION OF PLATE 1

- Fig. 1.—General layout of light screens and pots in experiment 4. The medium light-short day plants are in the foreground, and in the background from left to right are: low light-short day, low light-long day, high light-short day, and high light-long day plants. Two of the light-tight boxes used to cover the short day plants may be seen.
- Fig. 2.—From left to right—high light, medium light, and low light plants from experiment 4. The spindly growth of the low light plants and the short petioles of the high light plants contrast with the medium light growth.

EXPERIMENTS ON THE APPLICATION OF AUTORADIOGRAPHIC TECHNIQUES TO THE STUDY OF PROBLEMS IN PLANT PHYSIOLOGY

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Summary

A method of autoradiography has been developed which allows the record of single β -particles emitted from sections of plant tissue 5-7 μ thick. The method allows resolution of the order of 1 μ and magnification up to $\times 1500$. Dissolution of the water and alcohol soluble contents of the tissue has been overcome, and it is therefore possible to determine the distribution of compounds concerned in the metabolism and biosynthesis of a cell. Cellular distortion and loss of contents during fixation and photographic processing is illustrated, and it is hoped that an improved method of freeze-drying will reduce this limitation. In macroscopic autoradiography the soya bean has proved to be most valuable plant material, because the thin stems and leaves allow autographs which give a detailed record of the transported compounds containing ^{14}C . The isotope is introduced to the plant in the photosynthesis of $^{14}\text{CO}_2$, and after a suitable period, the plant is dissected, dried, and placed against X-ray film. After 14 days exposure the film is developed, and the photographic image records positions and relative concentration of the isotope in the plant tissue.

I. INTRODUCTION

The earliest experiments with radioactive tracers in plants were confined to a study of the macroscopic distribution of certain substances, such as phosphorus, which could be readily labelled, and successful autoradiographs of leaves and fruits were obtained (Arnon, Stout, and Sipos 1940; Colwell 1942; Harrison, Thomas, and Hill 1944; Grosse and Snyder 1947). In the present investigation an attempt has been made to develop techniques which will increase the application of ^{14}C autoradiography to allow more detailed study of problems in plant and animal physiology. Low resolution autoradiography using X-ray film has yielded interesting qualitative information, and a simple method for the administration of $^{14}\text{CO}_2$ to plants during a period of photosynthesis has been used. Electron-sensitive emulsions have been employed to obtain increased resolution, and a method has been developed of measuring low concentrations of active isotope in tissue in which the electron tracks due to the specimen are counted. The problems of maintaining intimate association between specimen and emulsion and preventing isotope dissolution during tissue preparation and photographic exposure have been considered, and methods

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obtained which considerably reduce isotope displacement without loss of resolution.

Davenport and Stevens (1954), in a paper illustrating the use of X-ray film for comparing radioactivities, claim that by visual density matching, estimates of activities can be made with tolerable accuracy. In their report, the advantages of simplicity of the X-ray film technique and the possibility for practical comparisons of very weak sources are recognized. The possibility exists of describing the distribution of an isotope within whole leaves, stems, and roots, and of making estimates of the activities. Thickness of tissue must be kept in mind when comparing stem, root, and leaf autoradiographs, as comparisons can only be made between tissues of similar thickness.

Attempts have been made to obtain quantitative information from autoradiographs. The concentration of α -particle emitters in tissue has been measured by a count of α -particle tracks originating from tissue sections in contact with nuclear track emulsions (Endicott and Yagoda 1947; Rotblat and Ward 1953). The principal uncertainty in the technique appears to be the estimation of the self-absorption of the specimen. The method is one of high resolution, since the point of origin of the tracks may be observed with high precision. In autoradiography with β -emitting isotopes, quantitative estimates of the relative concentrations of the isotopes in different regions of the specimen have been achieved by a count of developed grains in the corresponding regions of the photographic emulsion (Andresen, Chapman-Andresen, and Holter 1952). Absolute determinations of isotope concentration by the method of grain counting have been made by Doniach and Pelc (1950), who also estimated the radiation dose received by the tissue in the course of the experiment, and by Nadler and Bogoroch (1951). The method is laborious and subject to errors arising from variations in the development conditions and from pseudo-photographic effects. Dudley and Pelc (1953) have developed an apparatus for automatic grain counting. This eliminates personal errors and allows a considerable reduction in statistical uncertainties. The apparatus projects a minute spot of light, moving on a pattern resembling a television raster, on to the autoradiograph. When the beam is interrupted by a silver grain an electric pulse is produced which is amplified and then passed through a discriminator unit before being recorded by a mechanical register. The number of grains rendered developable by the passage of a single electron through the emulsion must be known before the results of grain counts can be interpreted in terms of the actual concentration of active isotope present in the specimen. This can be achieved by standardization against a source of known specific activity, provided self-absorption is equal in both cases. Boyd and Levi (1950) have described a method of ^{14}C β -track autoradiography and they point out possibilities for quantitative evaluation of autoradiographs.

II. β -PARTICLE DETECTION IN AUTORADIOGRAPHY

The observation of the macroscopic distribution of a radioactive tracer absorbed in plant tissue presents little difficulty. The tissue is placed against a

photographic plate, either in contact with the emulsion surface or separated from it by a thin protective layer, for a suitable period, after which the plate is developed and the image compared with the tissue structure. A convenient detector of the activity present is provided by X-ray film, which has a high sensitivity to β -particles and is easily handled and processed. Such film is not, however, well suited to the microscopic investigations of the distribution of an active isotope, since the large grain diameter, of the order of $2\ \mu$, results in poor resolutions. Improved resolution can be obtained by the use of fine-grain emulsions, and in particular with nuclear emulsion, in which the reduction in sensitivity to be expected from the much smaller grain size ($0.2\text{--}0.5\ \mu$) is partially offset by the much higher concentration of silver bromide.

The resolution of an autoradiograph is not solely determined by the grain size of the emulsion used, but depends also on the specimen thickness, the separation between specimen and emulsion, and the β -particle range. The dependence of resolution on these factors is discussed by Doniach and Pelc (1950) and by Herz (1951). A lower limit of the order of $3\ \mu$ is to be expected for a sample thickness of $5\ \mu$ with an emulsion $2\ \mu$ thick.

If thick emulsion layers are used, in which the tracks of the individual electrons may be observed, comparable resolution may still be obtainable, since the point of origin of the tracks in the emulsion can be observed to an accuracy of the order of the grain diameter, even though the resolution of the image as a whole is lower than in the case of a thin emulsion layer. Full advantage cannot be obtained from observation of individual tracks unless the specimen remains in contact with the emulsion surface throughout the processing, so that the points of origin of the electron tracks may be directly related to the specimen structure.

The electron-sensitive emulsion now available can record the passage of electrons at minimum ionization energy ($0.5\ \text{MeV}$), which produce tracks of approximately 30 developed grains per $100\ \mu$ path length. The tracks of high energy electrons are practically linear, but as the energy decreases, large-angle scatters become increasingly frequent and the greater ionization results in an increase in the number of grains per unit path length.

Since electron-sensitive emulsions record the passage of every ionizing particle they rapidly accumulate tracks due to cosmic radiation and local radioactive contamination. The rate of formation of such tracks in an unshielded emulsion, $200\ \mu$ thick, is of the order of 200 per sq.mm. per day. This track background, which is superimposed upon the image produced by the specimen, is frequently quoted as an objection to the use of such plates for autoradiography. It does not, in practice, limit the detection of low levels of activity too seriously if freshly prepared plates are used, since specific activities of the order of 10^{-9} c/g would yield electron tracks at a rate comparable with the formation of background tracks. If necessary the background accumulation rate can be reduced by shielding. If the plates are enclosed with a 2 in. lead wall and covered by a thickness of 4 in. of lead during the exposure, the background will be decreased by a factor of about nine (Fremlin and Walters 1950).

One of the advantages to be gained from the use of thick electron-sensitive emulsions is that quantitative estimates of the concentration of active isotope in the specimen may be made from a count of the electron tracks forming the autoradiograph. In such measurements the background of tracks due to other sources must be observed in a region of the plate protected from the active specimen, and the measured value of the sample emission corrected accordingly. Tracks caused by the emission of electrons from the specimen are easily distinguished from fog grains and pseudo-chemical effects.

Electron tracks, particularly those originating from an isotope of low disintegration energy, are not easy to count as a consequence of the wide variation in initial energy of the electrons producing them, and the frequency of large-angle scatters. If, however, the total number of tracks in 1 sq. mm. of the autoradiograph is kept below 500, and the tracks are observed with high power. oil immersion objective, repeatable counts may be made. The counting is simplified if only those tracks intersecting the upper emulsion surface are included in the total. This also effectively reduces the track background, since many background tracks are caused by electrons arising from the absorption of γ -radiation within the emulsion or glass backing, and do not penetrate to the emulsion surface.

In order to obtain an absolute estimate of the specific activity of the sample from the results of such a count, several factors such as self-absorption in the source, back-scattering in the emulsion, statistical uncertainties, and observational errors must be taken into account. The possibility of calibrating the detection system exists for the case of ^{14}C since A.E.R.E., Harwell, supply standard sources of polymethylmethacrylate sheet containing accurately measured concentrations of ^{14}C . These may be sectioned to the same thickness as the specimen. Counts of tracks produced by such a source allow the combined effects of self-absorption, back-scattering in the emulsion, and any failure to include the tracks of very low energy electrons in the count to be determined. The number of electron tracks observed within a definite area of an autoradiograph may then be related to the specific activity of the sample by the application of the laws of radioactive decay. If the isotope concerned has a half-life $T_{\frac{1}{2}}$, long in comparison with the exposure period t , the total number of electrons emitted n , is given by the relation

$$n = N \lambda t,$$

where N is the total number of radioactive atoms present in the sample, and λ is the decay constant of the isotope and is equal to $0.69/T_{\frac{1}{2}}$.

Only a certain fraction of the electrons emitted by the active isotope will enter the emulsion, this fraction being determined by self-absorption in the specimen and the geometry of the system. If a specimen, thin in comparison to the β -particle range, is exposed in contact with the emulsion surface, it may be assumed that one-half of the emitted electrons will be recorded in the emulsion. An error of less than 5 per cent. is involved for a sample thickness of one-tenth of the β -particle range. If a tissue density of 1 is assumed, a specimen $40\ \mu$ thick would allow half of the β -particles emitted to pass out of the tissue. As sections 5 and $7\ \mu$ thick are used it may be assumed that the

error due to self-absorption is small. When thicker sources are used, self-absorption becomes appreciable. The number of electrons which are able to reach the surface of a thick source of known specific activity has been estimated by Libby (1947) on semi-empirical grounds. Using the experimental fact that electrons from typical β -emitters are absorbed exponentially up to 90 per cent. loss of intensity, it is shown that the number of electrons reaching the surface of a source of infinite thickness is

$$I = \frac{A \sigma L_0}{2 \times L_0^a},$$

where I = intensity,

A = area of source,

σ = specific activity,

L_0 = β -particle range, and

a = absorption coefficient of the β -particles in the material of the source.

For many β -emitters the product $2 \times L_0^a$ is approximately equal to 10. Thus one-tenth of the β -particles originating in a layer of the source of thickness equal to the range are able to reach its surface.

For sources of thickness x (less than the β -particle range), the intensity I_x at the surface is related to the intensity I_∞ due to a source of thickness equal to or greater than the range by the expression

$$I_x = I_\infty (1 - e^{-ax}).$$

Values of the ratio I_x/I_∞ for different source thicknesses, expressed as a fraction of the β -particle range, have been calculated and are shown graphically in Figure 1.

The proportion of the disintegrations likely to be absorbed in any practical case may thus be estimated if the source thickness and β -particle range are known. Absolute measurements of the concentration of radioactive tracers in tissue may, therefore, be made with reasonable accuracy if thick nuclear emulsions are employed, the accuracy of the determination being limited primarily by uncertainties as to the amount of self-absorption occurring in the source and the detection efficiency. Statistical uncertainties may be reduced to a value small in comparison with other errors without undue effort. The method is one of very high sensitivity, a concentration of ^{14}C of only 5×10^{-10} g per g of tissue being readily detectable with unshielded plates.

III. EXPERIMENTAL PROCEDURE

(a) Administration of $^{14}\text{CO}_2$ to the Plant

$^{14}\text{CO}_2$ is introduced to the plant in photosynthesis, and suitable exposure apparatus is required for the control of this process.

One administration method allows control of the site of $^{14}\text{CO}_2$ assimilation and the use of intact plants. This requires a 10 c.c. weighing bottle with a sq.cm. opening at the bottom. The exposure chamber is attached to the abaxial surface of the leaf with porometer luting wax applied to the ground edge of the open square. Before attachment the required weight of

$\text{BaCO}_3/\text{Ba}^{14}\text{CO}_3$ (1560 : 1) is placed in the inverted weighing bottle to give a known total concentration of CO_2 . When adding carrier to obtain the required proportion great care must be exercised to obtain a uniform mixture. This has best been achieved by mixing in a small amount of carrier at a time and grinding the two together with a glass rod on a ground glass surface. At the commencement of the administration period, 0.2 ml (an excess) of N HCl is injected with a hypodermic syringe on to the BaCO_3 liberating a $\text{CO}_2/^{14}\text{CO}_2$ mixture (see Plate 1, Figs. 1 and 2). The abaxial side of the leaf is sealed on to the exposure chamber with the wax previously applied, and the

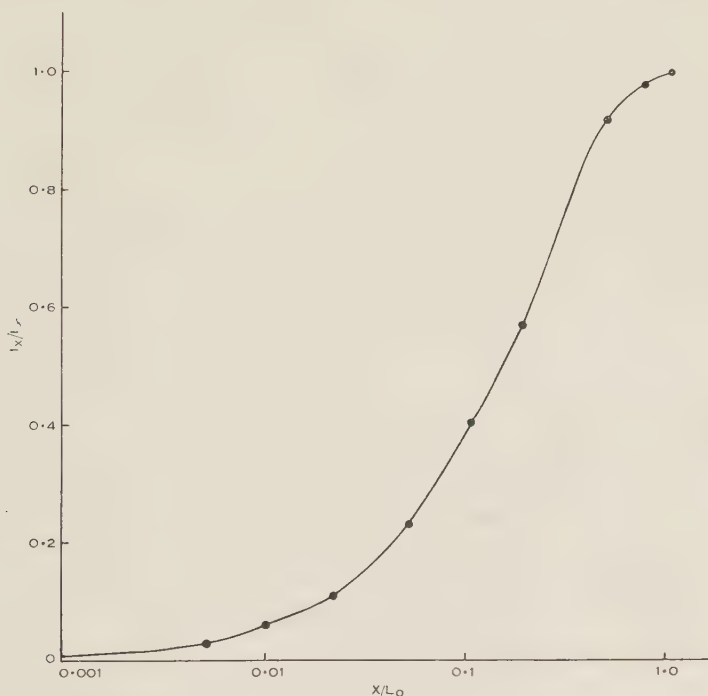


Fig. 1.—Values of the ratio I_x/I_∞ for different source thicknesses, expressed as a fraction of the β -particle range.

plant is allowed a period for photosynthesis. Most experiments were conducted in light intensities varying between 500 and 2000 f.c. At the end of the experiment the leaf is lifted quickly from the exposure chamber and replaced by a small piece of paper. In this way the amount of active gas escaping to the surrounding atmosphere is kept at a minimum. The plant is then dried in preparation for placing in contact with the photographic emulsion.

(b) Tissue Fixation and Dehydration

(i) *For Macroscopic Autoradiography.*—For the preparation of small tissue samples (i.e. a single leaf or seedling) used in the determining of macroscopic

distribution of ^{14}C , initial freezing is achieved by the immersion of tissue in an alcohol-dry ice freezing mixture, or for leaves, insertion in a Dewar flask containing crushed dry ice. Once frozen, the tissue is placed over a concentrated sulphuric acid trap in a vacuum desiccator attached to a high vacuum pump. A small quantity of dry ice is placed with the sample in the vacuum desiccator to prevent thawing before a high vacuum develops. This system achieves a pressure of 0.1-0.4 mm Hg, which gives satisfactory drying in 36-48 hr. Absolute dryness is not achieved by this procedure, but the moisture content of the tissue is reduced to a level satisfactory for X-ray autoradiography, and movement or displacement of isotope is insignificant when considered in relation to the resolving power and other limitations of macroscopic autoradiographs. When movement of the isotope into a particular tissue, as opposed to movement within a tissue, is the object of the experiment, air drying after dissection of the plant is satisfactory. Other methods of drying, including heat treatment of the tissue, cause considerable movement of the isotope in the vascular system of leaves and stem.

In the preparation of autoradiographs, the dried leaves, stems, and roots are placed on sheets of paper 5×7 in., their outlines traced, and other identification details noted (Plate 1, Fig. 3). Then the paper with specimens is placed between two sheets of $\frac{1}{8}$ in. glass of the same dimensions. Finally, in the dark-room, using a Wratten No. 1 filter, a piece of "Kodirex" (no-screen) duplitized X-ray film is placed in contact with the tissue, and the whole held firmly with spring-back clips. These plates are then deposited in a light-tight tin which is kept at $0-1^\circ\text{C}$ for 14 days. Variation of the exposure time presents a useful possibility for the comparison of tissue autographs and for activity estimations. Development is with Kodak X-ray developer for 3 min at 20°C , and the acid fixing solution, described below (see Appendix II(b)), is used after dilution with an equal volume of water. Contact prints are made from the autoradiographs, using light-weight glossy paper of high contrast, exposed 5 ft from an unshaded, frosted, 300-W, incandescent globe for 1-3 sec. Improved quality of autoradiograph prints can be achieved with photographic reduction.

(ii) *For Microscopic Autoradiography.*—Freeze-drying apparatus is used in tissue preparation for the purpose of obtaining autoradiographs of histological sections. The unit designed by Moberger, Lindstrom, and Andersson (1954) requires an oil diffusion pump and mechanical backing pump to reach a pressure of 10^{-4} - 10^{-5} mm. Hg. It is found that satisfactory preservation of plant tissue structure can be achieved with a simpler and less expensive design. The two vacuum pumps are replaced by a "Dynavac 6W" pump with a thermal stripping attachment, which prevents emulsification of the pump oil by water vapour from the specimen. Thus a water trap for the protection of the pump is not required. This pump will give a pressure of 10^{-3} mm Hg.

The tissue is placed on a piece of wire gauze supported by two blocks of degassed paraffin. Moberger *et al.* (1954) used an oscillator to heat the tissue holders within the tissue chamber, while it is found in this Laboratory that an infra-red lamp gives close control of paraffin melting. Well-preserved

tissue has been obtained without using a liquid air cold finger, but improvement might result from its inclusion, and drying time would doubtless be reduced. Greater detail of this method is to be published elsewhere. Immediately following exposure to the isotope, small tissue samples (total fresh weight not exceeding 100 mg) are quenched by direct immersion in liquid air, pre-cooled *isopentane*, or liquid propane. Specimens are then quickly transferred to the detached tissue chamber, which is a rigid fixture in a Dewar flask filled with alcohol-dry ice freezing mixture. The tissue chamber is fitted to the vacuum system and the pump switched on. Initial drying at a temperature lower than -60°C is allowed to continue from 6 to 12 hr, and then the Dewar flask is removed and the temperature of the tissue chamber allowed to equilibrate with air temperature. Pumping is continued for a further 12 hr. At the end of this period an infra-red lamp is brought in close proximity with the tissue chamber until the paraffin, above which the specimen has been placed, begins to melt, causing the collapse of the specimen stage and immersion of the specimen in the paraffin wax. The vacuum may now be broken and the impregnated specimen removed. The tissue at this stage is extremely brittle and must be handled with care. Paraffin blocks are trimmed to the required shape and size and microtome sectioning carried out in the usual manner. Sectioning should not be attempted until the photographic emulsion is poured and dried. The time lapse between section cutting and application to the emulsion surface must be kept to a minimum.

Hand sectioning of fresh material is a useful method of histological preparation. Stem sections are cut with a safety razor-blade in the darkroom and placed directly on to the emulsion surface. Good adhesion between the emulsion surface and the specimen is obtained without excessive chemical damage to the emulsion. An alternative would be to float the hand sections of living material on water and transfer these to the emulsion surface in the darkroom. Chemical fogging, if this occurs, is immediately distinguishable from the autoradiograph, which consists of β -particle tracks passing through the emulsion.

(c) *Spreading Paraffin Sections*

It has already been stated that one of the first requirements for the success of the microscopic technique is the prevention of isotope loss. Freeze-drying and paraffin embedding under vacuum give a ready means of tissue fixation with the possibility of only slight losses of lipoidal materials, but the spreading of sections presents a greater problem.

Sections 7μ thick are floated on a glycerine surface on a standard microscope slide, which is placed on an asbestos sheet over a water-bath (at approx. 40°C). Wetting of sections is kept to a minimum. The paraffin ribbon is divided into pieces containing two sections each, and the warmed slide transferred to the microscope stage and sections examined under the high-power lens. This examination allows an assessment of the quality of the section and cools the paraffin, which is necessary before further manipulation is possible. Once cool, the sections can be removed from the glycerine surface with the

aid of a camel-hair brush. This operation is not simple and will require practice before removal can be accomplished without damage to the sections. The aim is to remove the section with little or no glycerine adhering to its surface. Sections are placed on filter paper and the remaining free glycerine removed.

The glycerine film remaining is sufficient to hold the sections firmly on the emulsion surface, and when lightly wiped with a dry camel-hair brush provides an even and intimate contact throughout the exposure period. After photographic exposure, the plate is immersed in distilled xylol for approximately 2 min to remove paraffin from the sections before development. Using this method undamaged sections have adhered to the emulsion surface throughout photographic processing. Adjacent sections mounted in the usual way may be stained if greater detail is required for comparison with the section used to produce the autoradiograph. The staining of sections mounted on processed emulsion is possible, using alcoholic stains, but no great advantage results for leaf tissue. An experimental evaluation of isotope loss and displacement is presented in Appendix I.

(d) Photographic Processing

The electron-sensitive plates used in this investigation have been prepared in the laboratory, immediately prior to use, from G5 gel supplied by Ilford Ltd., England. Plate pouring avoids the unnecessary accumulation of background tracks, which would otherwise reduce the sensitivity of the method. The glass surface is first coated with a thin layer of a 5 per cent. solution of gelatine in water, which is poured at a temperature of 50°C over the glass supported on a cool, levelled table. When dry, the plate, a No. 1 coverslip $1\frac{1}{4} \times \frac{3}{4}$ in., is coated with G5 gel under a Wratten No. 1 safelight. The emulsion is warmed to a temperature of 55°C on a water-bath and 0.5 c.c. of the gel delivered to the surface of the plate with a warm, hypodermic syringe with no metal parts. An emulsion thickness of approximately 50 μ results. When set, the plate may be moved and dried in a stream of warm air at a temperature not exceeding 37°C. The best plates have been obtained when the humidity of the drying room was high, and the drying period extended over 12 hr. The thin gelatine layer is found to increase the adhesion between the glass and the emulsion, and to reduce reticulation during later processing. The coverslip may be more manageable if attached to the surface of a microscopic slide with a drop of water.

The development of nuclear track emulsions differs from that of optical emulsions, partly because of the greater concentration of silver bromide in the emulsion, and partly because the grains in which the latent image is formed are within and on the surface of the silver bromide crystals. An amidol-borax developer of the type described by Dainton, Gattiker, and Lock (1951) is used (see Appendix II(a)), and the "temperature development" method of Dilworth, Occhialini, and Payne (1948) employed in the case of emulsions of thickness greater than 50 μ , or on those sections which adhere to the emulsion surface. In this method the plate is soaked in the developer at a temperature below 5°C for a period of 3 min, during which time the developer penetrates

throughout the emulsion layer but the reaction is considerably retarded. The plate is then taken out of the solution, surplus developer removed from its surface, and placed glass downwards on to a hot plate at a temperature of 20°C for 20 min. Development then proceeds uniformly throughout the layer. On completion of the development the plate is transferred to an acid fixing bath (see Appendix II(*b*)) at 5°C, which terminates the development reaction while allowing the fixing solution to penetrate the emulsion. We have found it necessary, with plates prepared in this Laboratory, to complete fixation and washing at this low temperature in order to avoid reticulation. Acid fixing is continued for double the time taken for the plate to clear at 0-1°C. Washing is conducted at the same temperature, and both acid fixer and washing water are pre-cooled to approximately 5°C. Washing is continued until a sample of the water no longer reduces (pink to brown) potassium permanganate when a drop of a 1 per cent. solution is added. The plate is dried in a horizontal position in air, or dehydrated in a series of alcohol-water solutions of increasing alcohol concentration. The second method of drying is preferable in the case of plates carrying sections which are subsequently stained, since it results in more complete dehydration of the emulsion.

When dried, the coverslips, with emulsion and specimen attached, are inverted on to a drop of ethyl alcohol (previously dried with anhydrous copper sulphate) on a microscope slide. Using the oil immersion objective the β -particle tracks recorded in the emulsion can be associated with the specimen. With downward focusing the tracks are seen first and then the tissue.

IV. ILLUSTRATIONS OF MACROSCOPIC AND MICROSCOPIC AUTORADIOGRAPHS

This study was embarked upon with the primary purpose of finding a satisfactory experimental method for the study of translocation of organic materials in plants. The combination of macroscopic and microscopic autoradiography allows the record of the movement of assimilated isotope, and the results of experiments using these methods are to be published. Autoradiographs are presented here only to give illustration of the methods.

The G5 autoradiographs (e.g. see Plate 2, Fig. 6, and Plate 3, Fig. 7) illustrate the order of accuracy with which the activity can be located. The majority of sections remain undamaged throughout the photographic processing, and this arrangement allows detailed association of tissue structure with β -track origin at the emulsion surface. If the unstained section adhering to the emulsion surface is immersed in a drop of ethyl alcohol under a No. 1 coverslip, both section and autograph may be seen with a $\times 90$ oil immersion objective. Since, in the majority of cases, tracks enter the emulsion fairly steeply, only the first few grains are in focus in the photomicrograph. Single fog grains, which are readily distinguishable from tracks under working conditions, may be confused with the origin of β -particle tracks when viewed at a single focusing level; the photomicrographs, in which the tissue is slightly out of focus, are presented to illustrate the technique.

Plate 1, Figure 4, is a phase-contrast photomicrograph (magnification $\times 1300$) of the leaf cells and part of their autoradiographs. It is clear that very

close correlation between the tissue and emergent β -particle tracks is possible with this technique. A spongy mesophyll cell (X) in the centre of Plate 1, Figure 4, gives rise to five tracks originating in the centre of the cell and two tracks (of low energy and at a wide angle) from the cell wall. The disturbance of cell contents caused by photographic processing prevents the association of tracks with the structural features of the cells.

Plate 1, Figure 5, is presented to illustrate the record obtained of isotope distribution in G5 autoradiography, which immediately discloses if the isotope has moved from the tissue during preparation. From this autoradiograph of a transverse section of a *Cucurbita* petiole it can be seen that the isotope has moved into the embedding paraffin along the margin of the section, and it is reasonable to believe that similar movement occurred within the section. It is clear that such experimental errors are immediately evident, an attribute which aids accurate interpretation of the autoradiograph. This autoradiograph also illustrates that at lower magnification ($\times 480$) autoradiographs containing too many tracks for counting may be visually estimated, and so provide valuable information concerning the relative activities of different tissue regions. In the freeze-drying and subsequent processing, tissue damage has occurred and none of the cellular contents have been retained, but by using more efficient freeze-drying apparatus complete tissue preservation has been achieved.

Satisfactory microscopic autoradiographs have been prepared by cutting hand sections of fresh tissue containing assimilated ^{14}C . These fresh tissue sections are placed directly on to the surface of the G5 emulsion previously poured on to a coverslip. The objection to this method of tissue preparation is the possibility of redistribution of the isotope during sectioning. However, this possibility can often be ruled out because of the pattern and distribution of isotope recorded in the autoradiograph. The preservation of tissue structure and contents is satisfactory, and the use of hand sections should provide a useful application of the microscopic technique. Illustrations of the autoradiographs obtained from hand sections are presented in Plate 2, Figure 6 and Plate 3, Figure 7.

X-ray plate autoradiographs can record isotope distribution in whole plants and should, therefore, prove useful in the study of correlative nutritional relationships existing between plant organs. An experimental method is thus provided for the study of translocation of nutrients. Mineral uptake and distribution in plants may also be investigated using this method of autoradiography.

Examples of the macroscopic autoradiographs obtained are shown in Plate 3, Figures 8 and 9. In Plate 3, Figure 8, the movement of ^{14}C from the site of $^{14}\text{CO}_2$ administration in the leaf of soya bean up the stem to the apex and expanding leaf is recorded. No detectable autoradiograph is given by the opposite primary leaf, which is outlined to indicate its position on the X-ray film. The root autoradiograph (Plate 3, Fig. 9) shows accumulation of ^{14}C at the root tips, illustrating that the isotope incorporated in the products of photosynthesis moves from the illuminated primary leaf to these sites of meristematic activity and growth.

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EXPLANATION OF PLATES 1-3

PLATE 1

- Fig. 1.—Exposure chamber showing ground glass edge.
- Fig. 2.—Exposure chamber showing position of leaf on exposure to $^{14}\text{CO}_2$ during photosynthesis.
- Fig. 3.—Arrangement for X-ray plate exposure.
- Fig. 4.—Autoradiograph of several spongy mesophyll cells. Seven β -particle tracks are associated with the cell X in the centre of the photograph. Phase-contrast. $\times 1300$.
- Fig. 5.—Autoradiograph of a transverse section of a *Cucurbita* petiole produced from a 48-hr exposure. Note loss of isotope which occurred during embedding due to overheating paraffin. Tissue distortion and loss of cell contents are also evident. $\times 480$.

PLATE 2

- Fig. 6.—Autoradiograph of a longitudinal section of a soya bean stem. A pathway of active material across the sieve tube (X) can be detected from the line of β -particle tracks recorded in the overlying emulsion. Disturbance of the contents is indicated by the conglomerate in the centre of the sieve tube. β -particle tracks are also associated with the contents of the phloem fibres (Y). Phase-contrast. $\times 670$.

PLATE 3

Fig. 7.—Autoradiograph of a longitudinal section of the phloem of soya bean. A pathway of active material across the sieve tube (X) can be detected from the line of β -particle tracks recorded in the overlying emulsion. It will be noted that phase-contrast microscopy, used in Plate 2, Figure 6, gives improved association between the tissue and the autoradiograph. Oil immersion. $\times 1500$.

Fig. 8.—Autoradiograph recording the site of isotope administration, the pathway, and sites of accumulation of the products of photosynthesis, resulting from an exposure of the primary leaf of soya bean to $^{14}\text{CO}_2$ for 3 hr. $\times 1/3$.

Fig. 9.—X-ray film autoradiograph of part of a soya bean root following $^{14}\text{CO}_2$ administration to a leaf. $\times 1/3$.

APPENDIX I

(a) Isotope Loss and Displacement

Because of the basic importance of isotope loss or displacement to the success of the method, several plates were prepared for the measurement of activity levels as influenced by the time sections remained on a glycerine surface. Sections $7\ \mu$ thick were cut from the tip of a soya bean leaflet, and exposed to the nuclear emulsion for 144 hr. Table 1 gives results of track observations.

TABLE 1
INFLUENCE OF TIME OF SECTION ON GLYCERINE SURFACE ON TRACK DISTRIBUTION

Section No.	Time on Glycerine (min)	No. of Sections	No. of Fields	No. of Tracks per Field	Comment
1	2	1	15	22.6	Warmed twice
2	4	1	10	24.2	
3	4	1	10	22.2	
4	6	1	16	20.3	
5	6	1	10	23.3	Warmed twice
6	8	1	10	24.1	Warmed twice
7	8	1	10	25.6	Warmed twice
8	10	1	15	31.6	
Background			200	0.25	Marginal
4	6	1	33	0.79	
Mean per field				23.9	

The 33 fields around the margin of section No. 4 were counted for comparison with the background count. There are more tracks in this region than in the background, but the increase is small in comparison with the activity of the section. An increase of this magnitude could be accounted for by a residue of either adsorbed ^{14}C or isotopic lipoidal materials or both being displaced during paraffin embedding. The possibility remains that trace amounts escape from the section.

It can be seen from Table 1 that the track count does not decrease with time on glycerine over a 10-min period. Sections warmed twice do not show

a lower activity. On the contrary, there is a gradual rise of activity recorded for sections Nos. 5 to 8 inclusive, and section No. 8 shows a considerable increase. This rise recorded for section No. 8 is a conservative value, for in some areas of this section tracks were too numerous to count. The result suggests a displacement of ^{14}C within the section, when placed on a glycerine surface for 10 min and warmed twice, but evidently loss of ^{14}C to the glycerine does not occur.

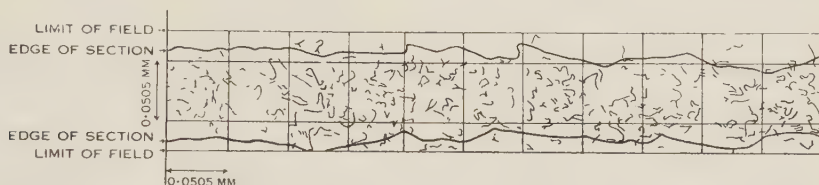


Fig. 2.—Distribution of β -particle tracks originating from a transverse section of soya bean leaf spread on anhydrous glycerine for 8 min and warmed twice.

A graphical picture of ^{14}C distribution within the soya bean leaf is shown in Figure 2. Relatively uniform distribution in mesophyll cells provides evidence that isotope displacement has not occurred. An uneven distribution would be expected if active materials had been moved from one part of the section to a new site, and such distribution was observed in section No. 8. The

TABLE 2

TRACK OBSERVATIONS TESTING THE CONSISTENCY OF TRACK COUNTING IN 16 SERIAL SECTIONS

Section	No. of Fields Observed*	Mean No. of Tracks Observed†	Section	No. of Fields Observed*	Mean No. of Tracks Observed†
A	55	1.8	J	18	2.0
B	20	1.7	K	13	1.4
D	19	1.3	M	54	1.3
E	21	1.7	N	29	1.2
F	17	1.5	O	24	1.1
G	23	1.8	P	57	1.3
H	60	1.3	Q	25	1.7
I	31	1.3	R	15	1.7
			‡Background	100	0.03

* Total number of fields observed = 481.

† Mean number of tracks per field = 1.5.

‡ Equivalent to 84 tracks per sq. mm.

small areas where activity is noticeably absent correspond to xylem tissue areas in that section. No tracks have been observed associated with xylem vessels, which again suggests that the isotope has not been displaced during processing.

To test the consistency of results that can be obtained from track counting when a very low concentration of ^{14}C is present in the tissue sections, a plate

was prepared from a masked region in the middle of the soya bean leaflet used previously. Of the 18 sections placed on the emulsion surface, 16 remained after processing. The damage to remaining sections varied, and the extent of this damage is indicated by the number of fields counted. The plate was exposed to sections for 92 hr. The result of this track counting is presented in Table 2.

The mean of such a large number of relatively uniform observations can be accepted as a valid representation of the ^{14}C present in the tissue.

When the activity of the two tissue samples exposed to $^{14}\text{CO}_2$ in the light (tip and base of leaflet) are compared on the basis of equal G5 exposure time, their ^{14}C concentrations fall within the same order of magnitude (i.e. base 17.2 and tip 23.9 tracks per $60\ \mu^2$). The apparently higher concentration in the leaf tip may be explained by the smaller area occupied by the xylem in this tissue sample. Compared on the same basis the tissue exposed to ^{14}C in the dark shows ^{14}C activity of 2.2 tracks per $60\ \mu^2$.

It is concluded that there is little or no loss or displacement of the ^{14}C isotope caused by spreading sections on anhydrous glycerine at 40°C for 2 min, and that, under the conditions of this experiment, similar concentrations of ^{14}C occur at the tip and the base of the Biloxi soya bean leaflet separated by a masked region after $1\frac{1}{2}$ hr of photosynthesis in an atmosphere containing $^{14}\text{CO}_2$.

APPENDIX II

(a) *Amidol-Borax Developer*

The composition of the amidol-borax developer is as follows:

Amidol	1.12 g
Sodium sulphite, anhydrous	4.5 g
Sodium sulphite, crystalline	9.0 g
Boric acid	8.75 g
Potassium bromide, 5 per cent. solution	4.0 ml
Water	to 250 ml.

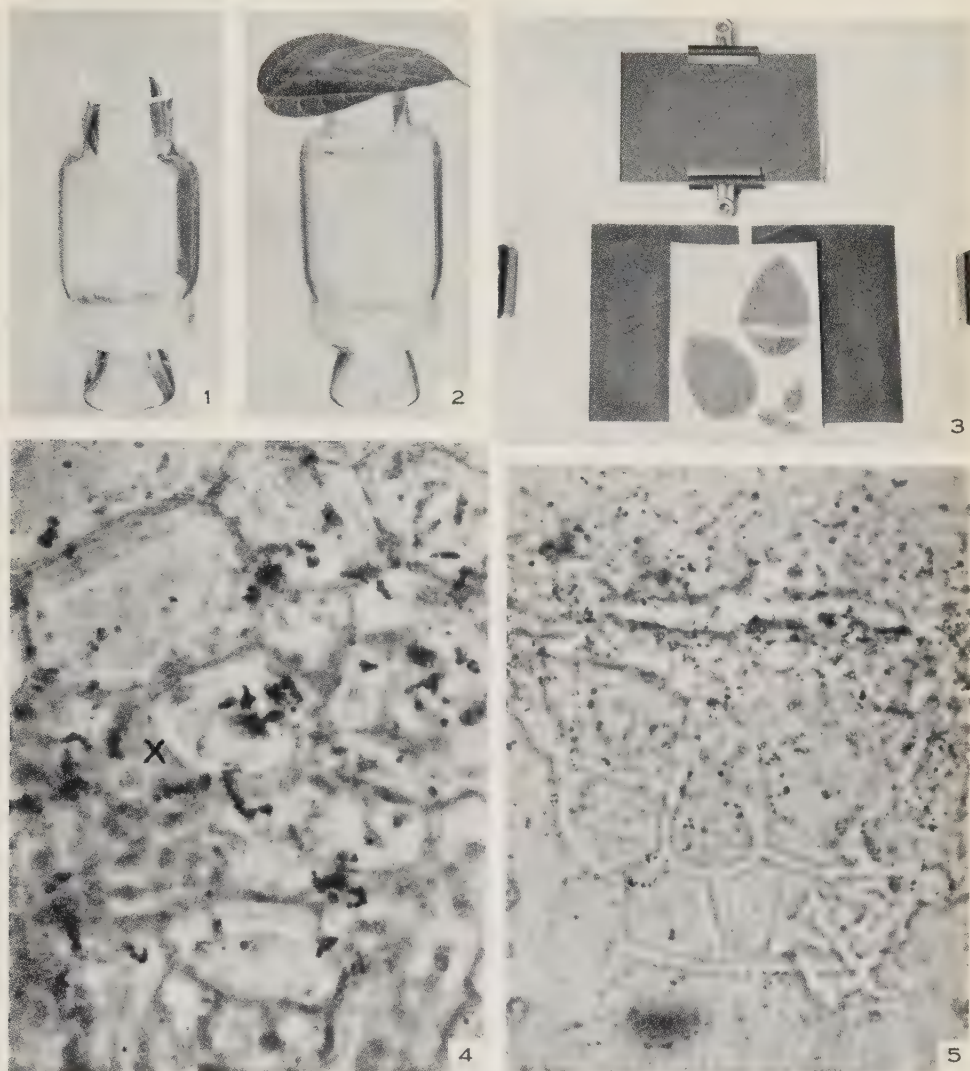
The sodium sulphite and boric acid are dissolved in 130 ml of distilled water at 50°C , then the amidol (dark crystals containing the product of oxidation are avoided) is dissolved in 80 ml of cold distilled water. The amidol solution is quickly filtered through cotton wool and the two parts mixed. Finally, the potassium bromide solution is added and made up to volume with distilled water. The amidol solution must not be allowed to discolour, otherwise stained plates and defective developing will result. This developer stored at 1°C can be used for 3-4 days after preparation.

(b) *Acid Fixing Solution*

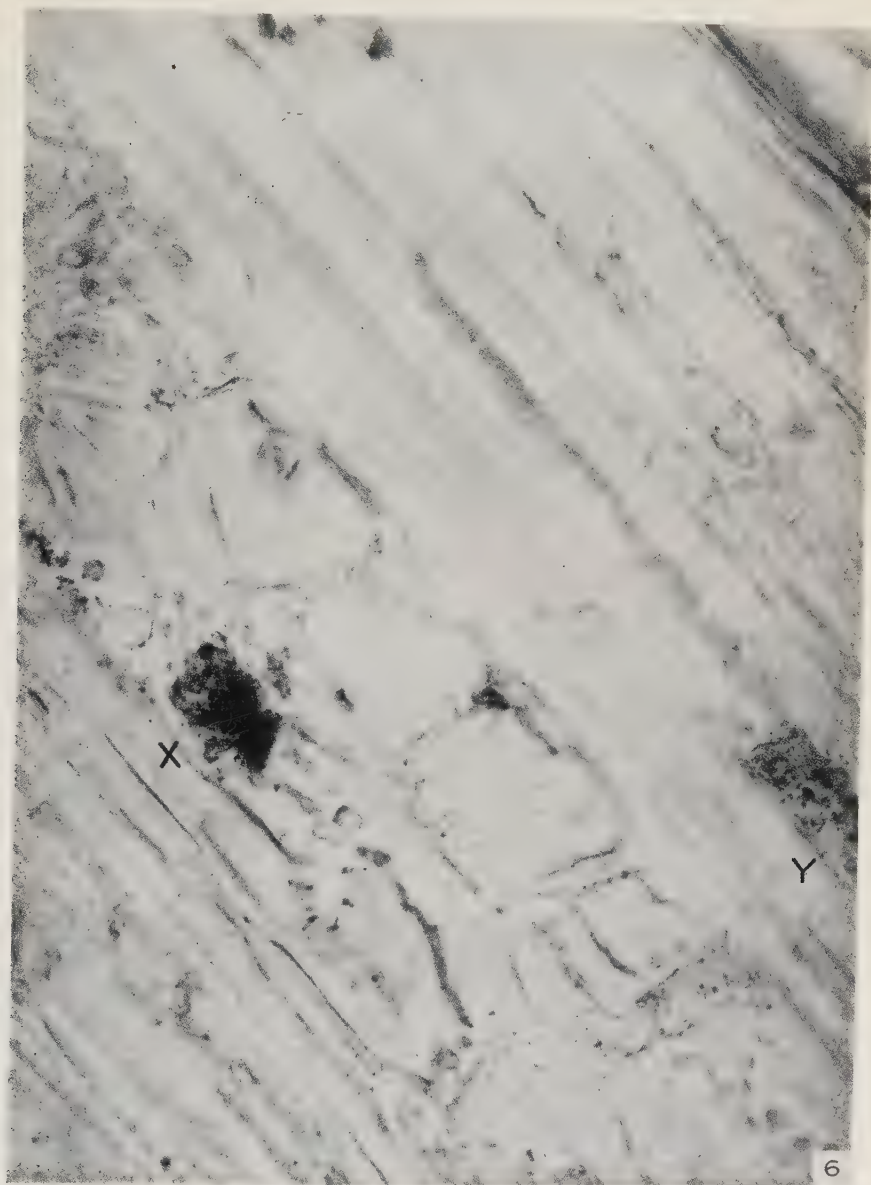
The composition of the acid fixing solution is as follows:

Sodium thiosulphate	400 g
Sodium bisulphite	30 g
Water	1000 ml.

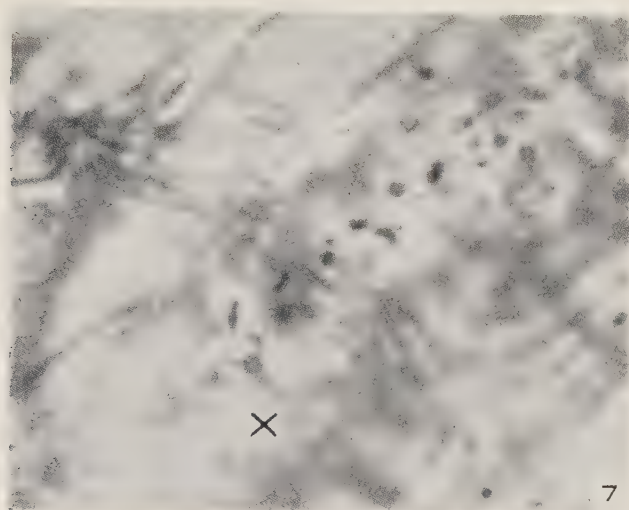
AUTORADIOGRAPHY FOR PLANT PHYSIOLOGY



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XANTHINE OXIDASE OF THE CLOTHES MOTH, *TINEOLA* *BISSELLIELLA*, AND SOME OTHER INSECTS

By H. IRZYKIEWICZ*

[Manuscript received March 15, 1955]

Summary

Xanthine oxidase activity in *Tineola* larvae averages 200 μ moles of uric acid per g whole larva (wet weight) per hr and in *Tenebrio*, *Lucilia*, *Anthrenocerus*, *Ephestia*, and *Anthrenus* larvae activity ranges between 13.4 and 1.3. The optimum pH for *Tineola* xanthine oxidase lies between pH 7.7 and 8.0, and the optimum concentration of xanthine is at or below 1.3×10^{-3} M. Methylene blue in concentrations up to 5.3×10^{-3} M has no toxic effect on this enzyme, and the lower concentrations of methylene blue have a limiting effect. Cyanide and 6-pteridyl aldehyde inhibit *Tineola* xanthine oxidase. The insect xanthine oxidases are demonstrated to be dehydrogenases. DPN, and pyruvate and DPN together, stimulate uric acid production by *Tineola* xanthine oxidase in the absence of methylene blue. In *Tenebrio* larvae there is a higher concentration of xanthine oxidase in the midgut and fat-body than in the remaining tissues.

I. INTRODUCTION

A very wide distribution of xanthine oxidase in the animal kingdom was demonstrated by Morgan (1926) and Florkin and Duchateau (1941). Larvae of *Tenebrio molitor* L. were shown to have outstandingly high activity, but no investigations on the properties of insect xanthine oxidase have been carried out.

Day (1951) reported the presence of xanthine oxidase in the gut of *Tineola bisselliella* (Humm.) larvae and suggested that the xanthine-uric acid system might be responsible for the maintenance of the very low oxidation-reduction potential found in the gut of this wool-digesting insect. It was apparent that more information was required on this enzyme, its properties, and distribution in insect tissues, before its role in the maintenance of the low oxidation-reduction potential could be evaluated.

Since the Thunberg methylene blue technique, as employed by Florkin and Duchateau (1941), and methods using tetrazolium salts (Anderson and Patton 1954) are liable to be affected by many different dehydrogenases in a crude enzyme solution, and since the hydrogen acceptor in the above mentioned methods is restricted to these dyes, a more specific method of measuring xanthine oxidase activity was adopted. This was based on the estimation of uric acid produced in the reaction mixture and by this method it has been shown that insect xanthine oxidases are true dehydrogenases and not oxidases.

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II. MATERIALS AND METHODS

(a) Reagents

The following reagents were used:

- 6.6×10^{-3} M xanthine solution (monosodium xanthine, Schwarz Laboratories),
- 0.1M tris(hydroxymethyl)aminomethane, buffer pH 7.9 ("Tris," Sigma Chemical Co.),
- 0.027M methylene blue solution (B.D.H.),
- 0.005M sodium cyanide (B.D.H.),
- 2-amino-4-hydroxy-6-formylpteridine,*
- 0.025M sodium pyruvate† (98 per cent. purity),
- Diphosphopyridine nucleotide (DPN) (Sigma Chemical Co.).

(b) Insect Tissue Homogenates

Preparations were obtained from larvae of *Tineola bisselliella*, *Tenebrio molitor*, *Lucilia cuprina* (Wied.), *Anthrenocerus australis* (Hope), *Ephestia kuehniella* Zell., and *Anthrenus flavipes* Le Conte by homogenizing tissues or whole insects in water or in saline solution in glass-"Teflon" tissue grinders at 0°C. With *Tenebrio* homogenates, very light centrifugation was applied to remove the larger fragments of hard cuticle. For the study of the distribution of xanthine oxidase, the midgut of *Tineola* and midgut and fat-body of *Tenebrio* were homogenized separately. To retain optimal conditions for uric acid estimation, a concentration of 25 mg tissue per ml of *Tineola* homogenate, 80 mg per ml of *Tenebrio*, and 100 mg per ml of other insects was required.

Dialyses were carried out against saline at 0°C with continuous mechanical stirring, renewing the saline every 20 min. After 2 hr 20 min, most of the endogenous uric acid had been removed, and after 3 hr the concentration of uric acid had fallen below the level at which it could be detected by the method employed for estimation. No loss of xanthine oxidase activity was caused by dialysis for 3 hr.

(c) Estimation of Xanthine Oxidase Activity

Activity of xanthine oxidase was measured by estimation of uric acid production from xanthine at 37°C. The reaction mixture contained 1 ml of insect homogenate, 1 ml of 6.6×10^{-3} M xanthine solution, 1 ml of 0.1M "Tris" buffer solution pH 7.9, 1 ml of 0.027M methylene blue solution, and 1 ml of water. When only molecular oxygen was provided as the hydrogen acceptor, water was substituted for the methylene blue solution. All solutions, with the exception of the homogenate, were pre-warmed to 37°C. After the addition of the homogenate, the tube was shaken thoroughly. Two 1 ml aliquots were immediately removed, and the tube was placed in a shaker apparatus in a water-bath at 37°C. The incubation period was governed by xanthine oxidase activity and varied from 30 min for *Tineola* to 2 hr for other insects. After the required incubation time, two 1 ml aliquots were again removed and pipetted

* Kindly supplied by Dr. D. B. Morell and † Dr. C. C. Kratzing.

directly into wide test tubes containing 1 ml of 10 per cent. sodium tungstate, 7 ml of water, and 1 ml of 2/3 N sulphuric acid, under which conditions protein was precipitated (Folin and Wu 1919). This treatment also caused precipitation of methylene blue. However, care was necessary to ensure that the filtrate

TABLE 1

XANTHINE OXIDASE ACTIVITY OF INSECT TISSUE HOMOGENATES AND URIC ACID CONTENT IN EXCRETA

Insect	Without Methylene Blue	With Methylene Blue		Uric Acid Content in Excreta (%)	
	Aerobic		Anaerobic		
μ moles Uric Acid per g Larva per hr					
<i>T. bisselliella</i>	6.5	200	200	31.7	
<i>E. kühniella</i>	0.4	1.8	2.5	—*	
<i>T. molitor</i>	0.5	13.4	13.4	—*	
<i>L. cuprina</i>	0	2.2	6.5	—*	
<i>A. australis</i>	0	2.9	4.0	8.7	
<i>A. flavipes</i>	0	1.0	1.3	1.1	

* No analyses carried out.

was completely clear and colourless since the methylene blue precipitate was very fine and mobile and had a tendency to creep around the filter paper when only small amounts of protein were present in solution. Uric acid was estimated by Newton's (1937) method, in 1 ml aliquots of the filtrate, using a Coleman

TABLE 2

EFFECT OF STORAGE ON *TINEOLA* XANTHINE OXIDASE ACTIVITY

Treatment	Loss of Activity (%)
Homogenate kept at 18°C for 24 hr	91.5
Homogenate kept at 0°C for 24 hr	13.0
Homogenate kept at 0°C for 120 hr	58.0
Homogenate kept at -22°C for 120 hr	0

Junior spectrophotometer for optical density readings at a wavelength of 680 m μ . Xanthine oxidase activity was expressed in μ moles of uric acid produced per g of wet insect tissue per hr.

For activity estimation under anaerobic conditions, Thunberg tubes were used. After aliquots at zero time were taken, the tubes were quickly evacuated, and twice flushed with oxygen-free nitrogen. After a further evacuation they were placed in a water-bath for incubation. The uric acid estimation was then carried out in the same way as for aerobic conditions.

Controls in which the xanthine solution was replaced by water were treated the same way as test estimations.

III. RESULTS

The activity of xanthine oxidase in *Tineola* larvae varied in different homogenates, but in all instances was very high, averaging 200 μ moles uric acid per g whole larvae (wet weight) per hr. Activity in other insects (Table 1) was of a much lower order and it ranged between 1.0 μ moles for *Anthrenus* and 13.4 μ moles for *Tenebrio*. In controls (omitting xanthine) for *Tineola* and *Lucilia* preparations no increase of uric acid was measurable, but for other insects uric acid produced in μ moles per g per hr ranged between 0.4 for *Tenebrio* and 2.5 for *Anthrenocerus*. The uric acid content in excreta of *Tineola*, *Anthrenocerus*, and *Anthrenus* (Table 1) is directly related to the xanthine oxidase activity of these insects.

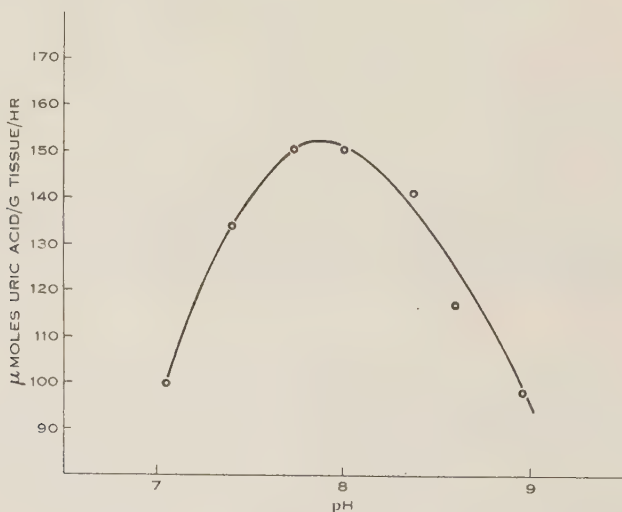


Fig. 1.—Effect of pH on xanthine oxidase activity of *Tineola* homogenate. Different pH values were established by addition of 1 ml of 0.1M "Tris" buffer of 7.9 pH range to the reaction mixture of final volume 5 ml. Incubated for 30 min at 37°C and uric acid measured.

Tineola homogenate lost its activity quite rapidly when kept at room temperature (about 18°C) (Table 2) but when stored in a refrigerator (about 0°C) for 5 days it retained 42 per cent. of the original activity. Homogenates which were deep-frozen (about -22°C) showed no decrease of activity after

5 days. The pH optimum for xanthine oxidase activity in *Tineola* preparations lies in a narrow range between pH 7.7 and 8.0 (Fig. 1).

High concentrations of xanthine have an inhibitory effect on *Tineola* xanthine oxidase and 27 per cent. decrease of xanthine oxidase activity was caused by the increase of xanthine concentration from $0.66 \times 10^{-3}\text{M}$ to $5.28 \times 10^{-3}\text{M}$ (Fig. 2, homogenate A) and 45 per cent. decrease was caused by increase of xanthine concentration from $1.3 \times 10^{-3}\text{M}$ to $15.8 \times 10^{-3}\text{M}$ (Fig. 2, homogenate B). The highest activity was obtained with a xanthine concentration of $1.3 \times 10^{-3}\text{M}$ and this concentration was chosen for further work.

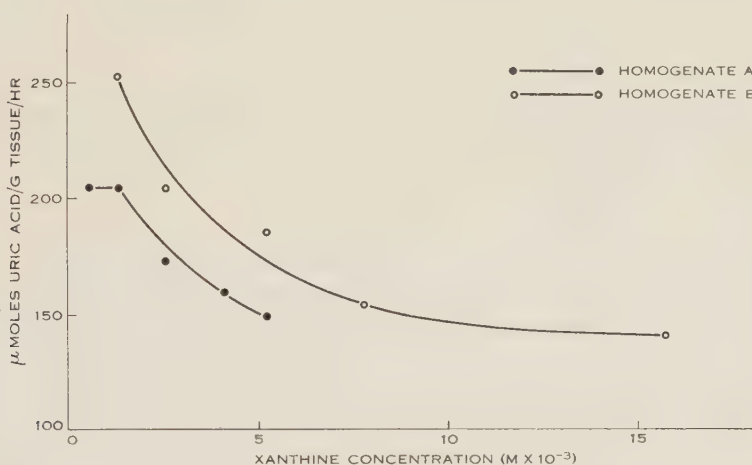


Fig. 2.—Effect of substrate concentration on xanthine oxidase activity of *Tineola* homogenates. Varying amounts of $13.2 \times 10^{-3}\text{M}$ or $39.6 \times 10^{-3}\text{M}$ xanthine solution and water were added to the reaction mixture to ensure final concentrations of xanthine of $0.66 \times 10^{-3}\text{M}$ to $15.84 \times 10^{-3}\text{M}$ and retain a final volume of 5 ml. Incubated for 30 min at 37°C and uric acid measured.

A very pronounced effect of methylene blue concentration on *Tineola* xanthine oxidase is demonstrated in Figure 3. The highest activity was measured when the methylene blue concentration was equal to or higher than $13.5 \times 10^{-4}\text{M}$ and activity dropped 48 per cent. when the methylene blue concentration was decreased to $8.4 \times 10^{-5}\text{M}$. A final concentration of $5.4 \times 10^{-3}\text{M}$ of methylene blue was used throughout this work. *Tineola* xanthine oxidase activity at this concentration of methylene blue lay on the plateau of the curve (Fig. 3) so that the dye produced no toxic effect. With this concentration of methylene blue, activities of the same order were obtained when estimations were carried out under either aerobic or anaerobic conditions.

Xanthine oxidase of *Tineola* homogenates was completely inhibited when these were pre-treated with sodium cyanide (Table 3). Addition of 2-amino-4-hydroxy-6-formylpteridine in a final concentration of 2 and $0.2 \mu\text{g}$ per ml to

the normal reaction mixture caused an inhibition of activity of 69 and 23 per cent. respectively.

TABLE 3
EFFECT OF CYANIDE AND 2-AMINO-4-HYDROXY-6-FORMYLPTERIDINE ON *TINEOLA* XANTHINE OXIDASE ACTIVITY

Compound Added to Reaction Mixture	Final Concentration of Compound Added	Inhibition (%)
Sodium cyanide	0.01 M*	100
Sodium cyanide	0.001 M*	100
2-amino-4-hydroxy-6-formylpteridine	2 μ g/ml	69
2-amino-4-hydroxy-6-formylpteridine	0.2 μ g/ml	23
2-amino-4-hydroxy-6-formylpteridine	0.02 μ g/ml	0

* Homogenate was pre-treated with sodium cyanide for 30 min at 37°C. In test without cyanide, homogenate was pre-treated the same way with water and the activity was 148 μ moles uric acid per g wet tissue per hr.

Sodium nitrate at 0.02M final concentration did not function as a hydrogen acceptor in the place of methylene blue under aerobic or anaerobic conditions. Similarly, a suspension of cystine in the presence or absence of DPN had no stimulating effect on uric acid production by *Tineola* homogenates. Replacing methylene blue solution with the same amount of 0.025M sodium pyruvate solution (final concentration 0.005M) did not cause any increase in activity of the *Tineola* homogenate. However, the addition of DPN at a final concentration of 8 μ g per ml produced some increase, and a still greater increase was stimulated by the addition of pyruvate together with DPN. A typical example of this effect is presented in Table 4. In controls with DPN plus pyruvate but without xanthine no increase in uric acid was found.

TABLE 4
EFFECT OF SODIUM PYRUVATE AND DPN ON URIC ACID PRODUCTION BY *TINEOLA* HOMOGENATE

Compound Added to Reaction Mixture	Final Concentration of Added Compound	Per Cent. of Activity*	
		Non Dialysed Homogenate	Dialysed Homogenate
Water		5.3	0
Sodium pyruvate	0.005M	5.3	0
DPN	8 μ g/ml	11.4	2.7
Sodium pyruvate + DPN	0.005M 8 μ g/ml	22.3	17.1

* As per cent. of activity of the same homogenate in presence of methylene blue at a final concentration of 5.4×10^{-3} M.

In the study of xanthine oxidase distribution in *Tineola* and *Tenebrio* larvae, homogenates of different tissues were prepared and the activity estimated. All calculations for distribution were made on the assumption that the midgut of *Tineola* constitutes about 10 per cent. of the wet weight of the intact larva; for *Tenebrio* the midgut forms 2.5 per cent. and the separated fat-body 10 per cent. of a whole larva. About 10.5 per cent. of total xanthine oxidase activity in *Tineola* larvae was recovered from the midgut and 84.5 per cent. was recovered from the rest of the body (Table 5). The loss of about 5 per cent. of activity is attributed to loss of tissue and some inactivation during the process of dissection. In *Tenebrio* larvae 8.6 per cent. of xanthine oxidase activity was found in the midgut, 33.6 per cent. in the fat-body, and 57.8 per cent. in the rest of the body.

TABLE 5

DISTRIBUTION OF XANTHINE OXIDASE IN TISSUES OF *TINEOLA* AND *TENEBRIO* LARVAE

Insect	Insect Tissues	Per cent. of Total Body Weight	Specific Activity (μ moles uric acid per g tissue per hr)	Per cent. of Total Activity in Larva
<i>T. bisselliella</i>	Midgut	10	198	10.5
	The remainder of the body	90	176	84.5
	Whole larva	100	188	100
<i>T. molitor</i>	Midgut	2.5	49.4	8.6
	Fat-body	10	48.2	33.6
	The remainder of the body	87.5	9.5	57.8

IV. DISCUSSION

The activity of xanthine oxidase in *Tineola* larvae is several times higher than that in rat and chicken liver. Williams and Elvehjem (1949) reported 190 μ l O_2 per g rat liver (wet weight) per hr, which corresponds to 8.5 μ moles of uric acid per g per hr, and van Pilsum (1953) 7.7 μ moles uric acid per g per hr. Morell (personal communication) has found for chicken liver that up to 50 μ moles uric acid per g per hr is produced.

The pH optimum for *Tineola* xanthine oxidase lies in a narrow range between pH 7.7-8.0 (Fig. 1), whereas that for crude milk xanthine oxidase was found to lie in a wide range pH 6 to 9 (Dixon and Thurlow 1924) and rat liver xanthine oxidase pH 8 to 9 (van Pilsum 1953).

High concentrations of xanthine have an inhibitory effect on *Tineola* xanthine oxidase and it appears that optimum xanthine concentration is not greater than $1.3 \times 10^{-3}M$ (Fig. 2). For milk xanthine oxidase the optimal xanthine concentration lies below $0.9 \times 10^{-3}M$ (Dixon and Thurlow 1924). Rat liver homogenate tolerated a much higher xanthine concentration, up to

$4 \times 10^{-2}M$, without any inhibitory effect on its activity (van Pilsum 1953). According to this author, however, a purified preparation of rat liver xanthine oxidase was quite rapidly inhibited when the concentration of xanthine was increased from $4 \times 10^{-3}M$ to $3.6 \times 10^{-2}M$ (about 90 per cent. inhibition). Xanthine oxidase of *Tineola* homogenates appears to be similar to milk and purified rat liver xanthine oxidase in its reaction to increased concentrations of xanthine, although the inhibitory effect was not as marked with the crude insect enzyme.

Methylene blue in higher concentrations (up to $5.4 \times 10^{-3}M$) did not have any toxic effect on *Tineola* xanthine oxidase and the relation of methylene blue concentration to xanthine oxidase activity resembles the relation of a substrate concentration to enzyme activity (Fig. 3).

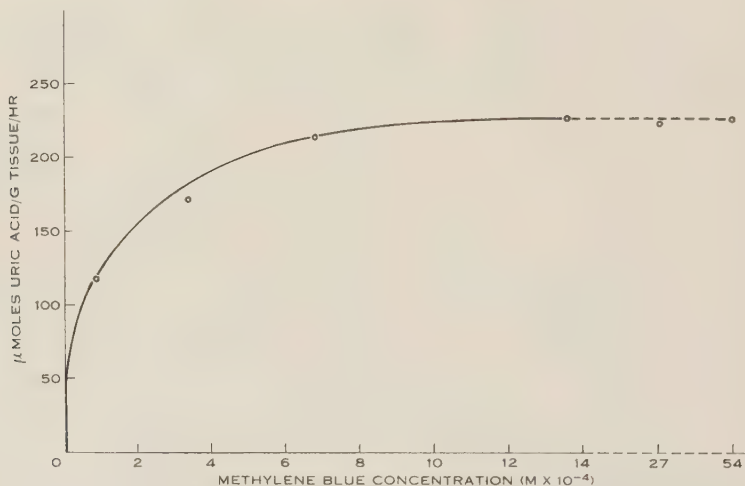


Fig. 3.—Effect of methylene blue concentration on xanthine oxidase activity of *Tineola* homogenate. Methylene blue solutions of varied concentrations were added to the reaction mixture to obtain final concentrations of methylene blue of $0.8 \times 10^{-4}M$ to $5.4 \times 10^{-3}M$. Incubated for 30 min at $37^{\circ}C$ and uric acid measured.

The inhibition of *Tineola* xanthine oxidase by pre-treatment with cyanide (Table 3) is of the same character as the corresponding inhibition of milk xanthine oxidase (Dixon and Keilin 1936). Kalckar, Kjeldgaard, and Klenow (1948) have demonstrated a very potent inhibitory effect of 2-amino-4-hydroxy-6-pteridyl aldehyde on xanthine oxidase. This inhibitor had a similar, but less pronounced, effect on *Tineola* xanthine oxidase.

When methylene blue was omitted from the incubation mixtures, the activity of xanthine oxidase in insect homogenates dropped to a very small fraction of the previous value or disappeared completely (Table 1). Furthermore, after 3 hr dialysis of *Tineola* homogenate, no activity could be found in the absence of methylene blue (Table 4). This suggests that insect xanthine oxidases are dehydrogenases. Richert and Westerfeld (1951) demonstrated that pigeon kidney and chicken and turkey liver homogenates oxidize xanthine to uric acid

at a very low rate when molecular oxygen only was offered as the hydrogen acceptor, and that the addition of methylene blue increased several fold the rate of this oxidation. Morell (1955) has shown that methylene blue increased uric acid production by chicken liver xanthine oxidase about 12- to 16-fold. The same author has also demonstrated stimulation of chicken liver xanthine oxidase activity by DPN, pyruvate, D-ketoglutarate, and fumarate. DPN alone and pyruvate plus DPN increased the production of uric acid by *Tineola* homogenates (Table 4), whereas pyruvate without DPN had no effect at all. This suggests that the level of DPN concentration in *Tineola* larvae is very low or that destruction of DPN occurred in the preparation of the homogenates. The stimulating effect of DPN and pyruvate on *Tineola* xanthine oxidase is interesting in view of the suggestion (Morell 1955) that xanthine oxidase in chicken liver is linked to lactic dehydrogenase through DPN. On the other hand, if DPN is directly reduced by xanthine oxidase in the presence of xanthine and if the concentration of DPN of $8 \mu\text{g}$ per ml ($1.2 \times 10^{-5}\text{M}$) is sub-optimal for this reaction, the addition of pyruvate in the presence of lactic dehydrogenase would increase DPN and thus increase uric acid production.

Only a small fraction of the xanthine oxidase activity is located in the gut of *Tineola* (10.5 per cent.) and *Tenebrio* larvae (8.6 per cent.), although the gut is as active as any other tissue examined. In *Tenebrio* larvae 33.6 per cent. of xanthine oxidase was found in the fat-body, but undoubtedly this figure is too low, as some of the fat-body could not be separated from the rest of the body. Specific activities of midgut and fat-body tissue of *Tenebrio* are of much higher order than the specific activity of the remaining tissues (Table 5) and this is in agreement with the findings of Leifert (1935) and Anderson and Patton (1954).

V. ACKNOWLEDGMENTS

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AN ELECTROPHORETIC STUDY ON "COMPONENT 2" EXTRACTED FROM WOOL WITH ALKALINE THIOLYCOLLATE

By J. M. GILLESPIE* and F. G. LENNOX*

[Manuscript received March 18, 1955]

Summary

Solutions of the thioglycollate-reduced wool keratin preparation, "component 2" of Gillespie and Lennox (1953, 1955), show abnormal electrophoretic behaviour. New, faster moving peaks appear in the descending electrophoretic pattern at protein concentrations exceeding 0.5 per cent. which are attributed to an aggregation-disaggregation reaction. They are eliminated by increasing the ionic strength to 0.5, or by lowering the protein concentration to 0.4 per cent.

The protein is modified by storage, both in solution and in the solid state, the modification being favoured by increase in temperature and manifested by a fall in relative viscosity of the solution together with a corresponding increase in mobility. The mobility increases only slightly as the pH is raised from 7 to 11, but it increases steeply at higher pH values.

The intrinsic viscosity is about 0.2 in the pH range 8-10.

I. INTRODUCTION

The preparation from Merino 64's wool of an electrophoretically pure fraction representing the major protein in wool and designated "component 2" was recently described by Gillespie and Lennox (1953, 1955). It was shown to have an apparent molecular weight approximating 32,000 at pH 6.9 and $1.0\Gamma_{1/2}$ but at pH 9.0 dissociates to units of molecular weight approximating to 8000 at $0.01\Gamma_{1/2}$ (Harrap 1955*b*). It contained less cystine but more glutamic acid residues and amide groups than the wool from which it was derived (Simmonds 1955). Under a variety of conditions it moved as a single peak in the ascending limb of the electrophoresis apparatus but showed a multiple peak in the descending limb at protein concentrations exceeding 0.5 per cent.

In the present study this interesting electrophoretic behaviour is further investigated and the influence of several factors on the mobility of component 2 is reported.

II. MATERIALS AND METHODS

The protein used in the present investigation, component 2, was prepared as previously described (Gillespie and Lennox 1953, 1955) and run electrophoretically for approximately 6 hr at 1°C. Briefly, solvent-extracted wool was extracted five times for 20 min at 50°C with 0.1M thioglycollate at an initial pH of 10.5 using 30 ml for each g wool. The residue was extracted once with 0.1M thioglycollate at an initial pH of 12.3 to yield a solution containing only

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component 2. This extract was either freeze-dried direct or precipitated with acetic acid, redissolved in alkaline thioglycollate, and then freeze-dried. Viscosity measurements were made at 25°C in an Ostwald viscometer with time of flow for water at 25°C of about 200 sec.

III. RESULTS

(a) Factors Influencing the Electrophoretic Pattern

(i) *Protein Concentration.*—Thioglycollate-glycine buffer (Gillespie and Lennox 1953) at pH 11 and $0.22 \Gamma_2$ and containing protein at concentrations ranging from 0.3 to 1.8 per cent. was used. The ascending boundary showed

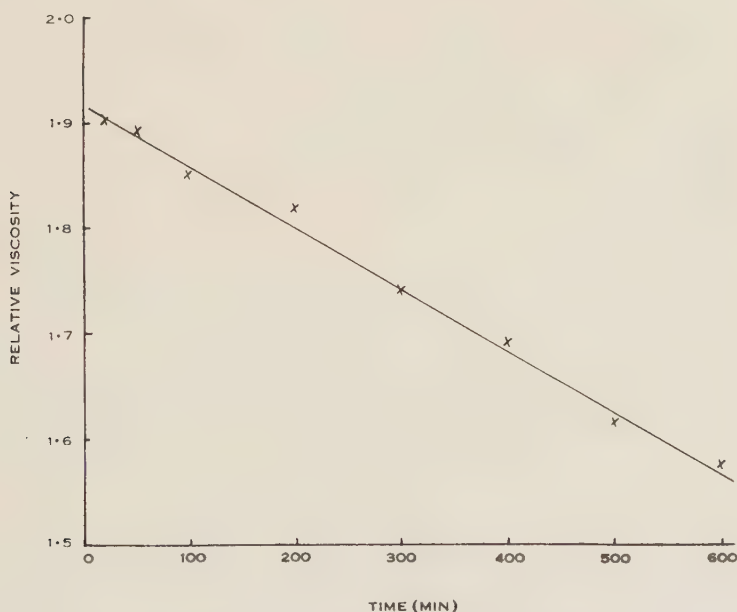


Fig. 1.—Decrease in relative viscosity of a 1.6 per cent. solution of component 2 over 10 hr at 25°C at pH 10.0.

an apparently normal, single, symmetrical peak at all protein concentrations, and in the descending boundary a single, symmetrical curve was obtained at a protein concentration of 0.3 per cent. A slight hump on the curve, attributable to faster moving material, was observed at 0.6 per cent. protein concentration and, as this increased, so also did the amount of faster moving material (Plate 1).

(ii) *Buffer Concentration.*—Solutions containing 1.5 per cent. protein were run at pH 11 in a series of buffers containing thioglycollate and glycine at $0.02 \Gamma_2$, that is at one-tenth the usual concentration, together with sufficient KCl to bring the final ionic strength to desired values within the range 0.02–0.5.

The two boundaries showed markedly different behaviour. In the descending boundary at the lowest ionic strengths, in addition to the main peak, a long hump of faster moving material showing no resolution into individual peaks can be seen in the patterns (Plate 2). As the ionic strength was increased, the amount of protein in the faster moving peaks decreased until at $0.5\Gamma/2$ the pattern assumed the form of a single, symmetrical peak, whereas the ascending boundary was hyper-sharp at the lowest ionic strengths, becoming normal at ionic strengths of 0.1 and higher.

(iii) *pH Value*.—Electrophoresis at lower pH values also revealed anomalous peaks in the pattern for the descending limb but they were less pronounced.

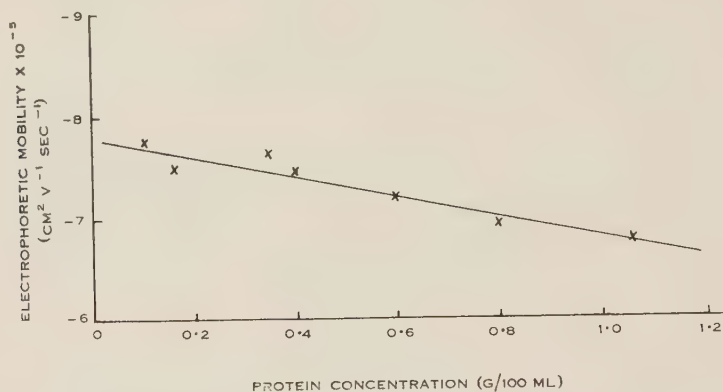


Fig. 2.—Relationship between protein concentration and electrophoretic mobility of component 2 run in buffers at pH 10.0 for 6 hr at 2.5 V cm^{-1} .

(b) Factors Influencing the Mobility

In our earlier studies, the electrophoretic mobilities determined under standard conditions for different preparations of component 2 showed a fairly wide scatter around a mean value of $-7.2 \times 10^{-5} \text{ cm}^2 \text{V}^{-1} \text{sec}^{-1}$ at pH 11 with a protein concentration of 0.5 per cent. In the following sections it will be shown that the observed variation between -6.9 and $-7.5 \times 10^{-5} \text{ cm}^2 \text{V}^{-1} \text{sec}^{-1}$ can be explained by the apparent instability of the wool protein in alkaline solution, a steady decrease in viscosity with time corresponding with an increase in mobility.

(i) *Influence of Time on the Viscosity*.—Freshly prepared component 2 was chilled to 1°C and dialysed against 0.02M thioglycollate at pH 10 for 24 hr, then placed in an Ostwald viscometer at 25°C . It can be seen from Figure 1 that the relative viscosity diminished steadily throughout the 600 min test period.

(ii) *Influence of Time and Temperature of Storage on Mobility*.—Freshly prepared component 2 was chilled to 1°C and dialysed against a glycine-thioglycollate buffer at pH 11 and $0.22\Gamma/2$. After dialysis for 2 days the

dialysate was divided into two parts, one being maintained at 1.0°C and the other at 25°C for 2 days. The relative viscosity at 25°C of the portion held at 1°C was 1.36 and of that held for 2 days at 25°C was 1.24. Mobilities determined on the two solutions were -6.7 and $-7.5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$ respectively at pH 11. Further evidence of an increase in the negative mobility of this protein during storage was obtained by repeated electrophoresis of the same sample. After each run the protein solution was recovered from the cell, dialysed at room temperature, freeze-dried, and redissolved for the next experiment.

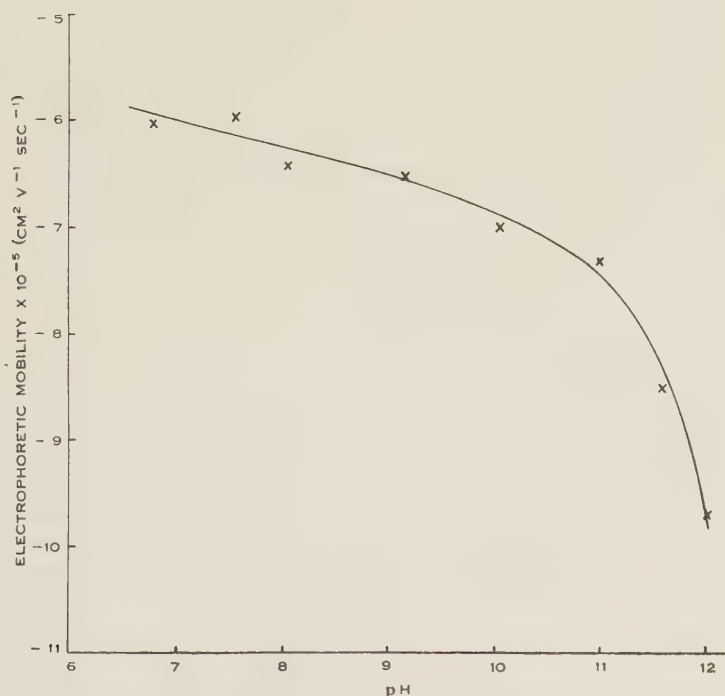


Fig. 3.—Effect of pH on electrophoretic mobility of component 2 at 0.3 per cent. protein concentration, run for 6 hr at 2.5 V cm^{-1} in buffers of ionic strength 0.2.

(iii) *Protein Concentration*.—The considerable effect of protein concentration on the mobility of component 2 already reported by the present authors was confirmed in these experiments. It was submitted to electrophoresis in a buffer at pH 10 and $0.2 \Gamma_{1/2}$ containing 0.02M thioglycollate, 0.02M glycine, and KOH and KCl to provide the required pH and ionic strength. The protein concentration tested covered the range 0.2–1.2 per cent. It can be seen from Figure 2 that the mobility of the protein is very dependent on concentration.

(iv) *pH Value*.—The pH-mobility relations of component 2 were mostly determined over the pH range 6.8–12.0. pH values below 6.8 were difficult to explore because of the very low solubility of the protein. The electrophoresis

equilibration solution contained 0.02M sodium thioglycollate, an appropriate buffer (0.02 $\Gamma/2$) to give the required pH, and sufficient KCl to bring the total ionic strength to 0.2 (Miller and Golder 1950). The protein concentration was 0.3 per cent. It can be seen from Figure 3 that there was a fairly sharp decrease in mobility as the pH was reduced to 11, and a much lesser change between pH 11 and 7.

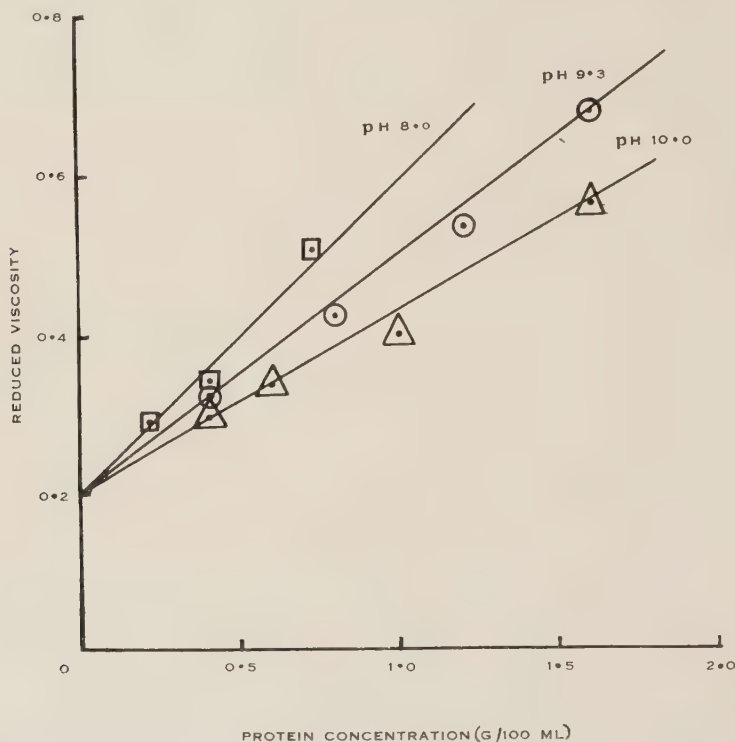


Fig. 4.—Relationship between reduced viscosity and protein concentration of component 2 at three pH values.

The mobility of component 2 at pH 4.0 was determined at a protein concentration of 0.1 per cent., in an acetate-thioglycollate buffer of ionic strength 0.2. It was found to be about $+0.4 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$ showing that at this pH the protein was on the acid side of the isoelectric point. It is of interest to note that Goddard and Michaelis (1935) suggested that the isoelectric point of an unfractionated thioglycollate extract of wool lay within the pH range 4.6-4.9.

(c) *Intrinsic Viscosity*

A freshly prepared solution of component 2 was dialysed at 1°C against 0.1 $\Gamma/2$ buffer (Miller and Golder 1950) containing 0.02M thioglycollate in the pH range 8-10 and stored at the same temperature and the viscosity measurements were made at 25°C. The viscosities of these solutions diminished during

incubation at 25°C but the first and second measurements required only 8 min during which the time of flow fell by only 1 or 2 sec in about 300 sec. The mean of these readings was used to calculate the reduced viscosities which are plotted against the concentration in Figure 4.

Over the pH range studied the reduced viscosity extrapolated to infinite dilution appeared to give the same value within the limits of experimental error.

(d) *Partial Specific Volume*

The value of the partial specific volume of component 2 was needed for the calculation of intrinsic viscosity; as no experimental measurements have been made, it was estimated from the amino acid analyses of Simmonds (1955) using the procedures described by Cohn and Edsall (1943). Recently McMeekin and Marshall (1952) have shown with 19 proteins that the calculated and experimental values are generally in excellent agreement. The partial specific volume of component 2 determined in this way was found to be 0.7175 and this value was used for the calculation of intrinsic viscosity. Olofsson and Gralén (1947) reported a value of 0.703 for an unfractionated sodium sulphide extract of wool.

IV. DISCUSSION

Component 2 shows peculiarities in its electrophoretic behaviour which make the interpretation of the results very difficult. New, faster moving components appear in the descending pattern with increase in protein concentration or with decrease in ionic strength, whereas the ascending boundary pattern shows no change with variation in protein concentration but becomes sharper with decrease in ionic strength.

This non-identity of boundaries has been observed with other proteins: with β -lactoglobulin (Ogston 1953; Smithies 1954) and with bovine plasma albumin at pH 4.63 (Longsworth and Jacobson 1949), at pH 4.15 (Alberty 1949), and at pH 2.2 in glycine-HCl at $0.1\text{ } \Gamma_{1/2}$ and also in fluoride-phosphate buffer at pH 6.8 (Woods, unpublished data).

Non-identity of ascending and descending electrophoresis boundaries can be caused either by protein-buffer interaction or by protein-protein interaction either between two different proteins or by aggregation of one protein. Protein-buffer interactions are almost certain to take place, for component 2 constitutes a reversible oxidation-reduction system in equilibrium with thioglycollic acid and its oxidation products, and any pH changes across boundaries could influence the relative proportions of -SH and -S-S- and thereby the charge on the protein at pH values where -SH is ionized.

There is no conclusive electrophoretic evidence that component 2 is one homogeneous protein. However, attempted fractionation did not resolve it into components, and O'Donnell and Woods (1955) found that the sedimentation pattern of a freshly prepared extract consisted of one well-defined peak when run in buffer at pH 11.0 and ionic strength approximately 0.3. The dependence of the electrophoretic patterns on protein concentration seems to indicate that the system consists of a monomer in equilibrium with its aggregates, and this is

supported by the findings of Harrap (1955*a*, 1955*b*). He showed by the surface balance technique that at pH 9, the molecular weight varied from 8000 at $0.01 \Gamma/2$ to 16,000 at $1.0 \Gamma/2$, whilst at pH 7, the molecular weight was 32,000 at $1.0 \Gamma/2$ and, contrary to the behaviour at pH 9, the protein became greatly associated at lower ionic strengths. This protein-protein interaction will be concentration dependent, and at any protein concentration there will be an equilibrium mixture of monomer and its aggregate. In order to explain the electrophoretic data it is assumed that the dimer and higher polymers migrate more rapidly than the monomer.

In the migration of an equilibrium mixture, the type of pattern obtained depends largely on the time of equilibrium. This has been discussed by many workers (e.g. Ogston 1953). The observations on component 2 seem to indicate that the time of equilibrium is of the same order as the time of electrophoretic separation. As Longworth and Jacobsen (1949) pointed out, in connection with serum albumin studies, an adjustment of equilibria will take place as electrophoretic separation occurs. Ogston (1953) has described such a case with β -lactoglobulin, namely, a hyper-sharp front on the ascending limb and an incompletely resolved descending boundary. This is attributed to the formation of a dimer which migrates more rapidly than the monomer. Some such explanation may be applied to the electrophoretic pattern of component 2.

At low protein concentrations, both boundaries appear to be symmetrical peaks and this may result from the dependence of the equilibrium reaction between monomer and dimer on the second power of the monomer concentration, or on higher powers if polymerization proceeds further. Hence at low concentrations very little of the aggregate would be present, but as the total protein concentration and hence the monomer concentration is increased there is a rapid increase in the proportion of dimer. Thus at low protein concentrations the dimer concentration may be too low to be resolved optically and also resolution may be blurred by more rapid diffusion.

The striking effects of ionic strength may be attributed to the fact that at low ionic strengths proteins move faster and heterogeneity is easier to detect; thus various effects due to the aggregation will be accentuated. However, the fact that the state of aggregation of the protein itself may depend on ionic strength should not be overlooked. This could account for the extremely hyper-sharp ascending boundary and for the descending boundary with a greatly spread leading edge obtained at the lowest ionic strengths. If the ideas of Harrap apply to component 2 in solution, then at the highest ionic strength only polymers will be present, and under these conditions both boundaries show a reasonably symmetrical peak.

None of the deviations from ideality described in the literature exactly meet the case of component 2 and a complete explanation of the observed effects cannot be given at the present time, but it seems that association-dissociation phenomena play a most important part in determining the shape of the patterns.

The results presented also indicate that component 2 constitutes an unstable system and the absolute values of mobility and viscosity reported here depend on the previous history of the keratin preparation. The chemical or physical

changes which take place in the protein during storage are not understood; it is quite likely, however, that oxidation is the predominant factor and this can almost certainly account for part of the changes in viscosity with time, but loss of amide groups or slow hydrolysis of other bonds cannot be excluded as causes of instability of the system.

Decrease in viscosity with time has been observed by Olofsson and Gralén (1947) with sodium sulphide extracts of wool, and also by Mr. E. F. Woods (personal communication) in this Laboratory working with peracetic acid extracts of wool. Such changes may therefore be a property characteristic of wool proteins.

In general the shape of the pH mobility curve resembles the general form of the titration curve of wool (Speakman 1953) in that at pH values above 11 there is a rapid increase in mobility due to discharge of the cationic amino acids with consequent increase in the net charge of the protein molecule. In dealing with an -SH protein it would be expected that there would be an increased net charge in the pH 9 region, because of the ionization of the -SH groups. However, the pH-mobility curve is not sufficiently accurate to provide information on this point. The pronounced insolubility of this protein in the isoelectric region makes it impossible to determine the isoelectric point by electrophoretic measurement and all that can be said is that it is slightly above pH 4 and well below pH 6.8.

V. ACKNOWLEDGMENTS

Thanks are due to Miss J. I. Meakin for carrying out the electrophoretic runs described in this paper, and to Mr. E. F. Woods and Mr. I. J. O'Donnell for helpful discussions on the theoretical aspects of the paper.

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

Influence of protein concentration on the electrophoretic patterns of component 2 run at 2.5 V cm^{-1} for 6 hr in buffers at pH 11 and ionic strength 0.2.

PLATE 2

Influence of ionic strength on the electrophoretic patterns of component 2 run at 2.5 V cm^{-1} for 6 hr in buffers at pH 11.

ELECTROPHORESIS OF WOOL PROTEIN

PROTEIN
CONCENTRATION
(%)DESCENDING BOUNDARY
←→
ASCENDING BOUNDARY

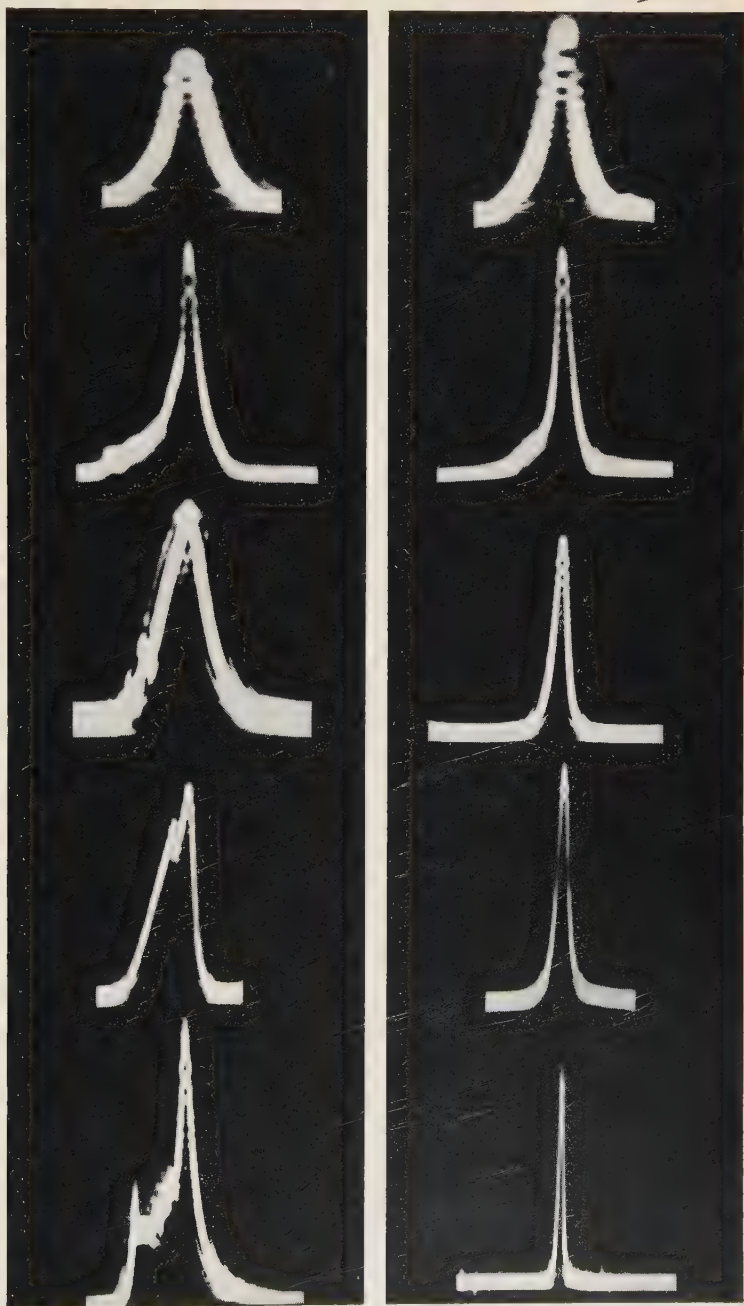
0.3

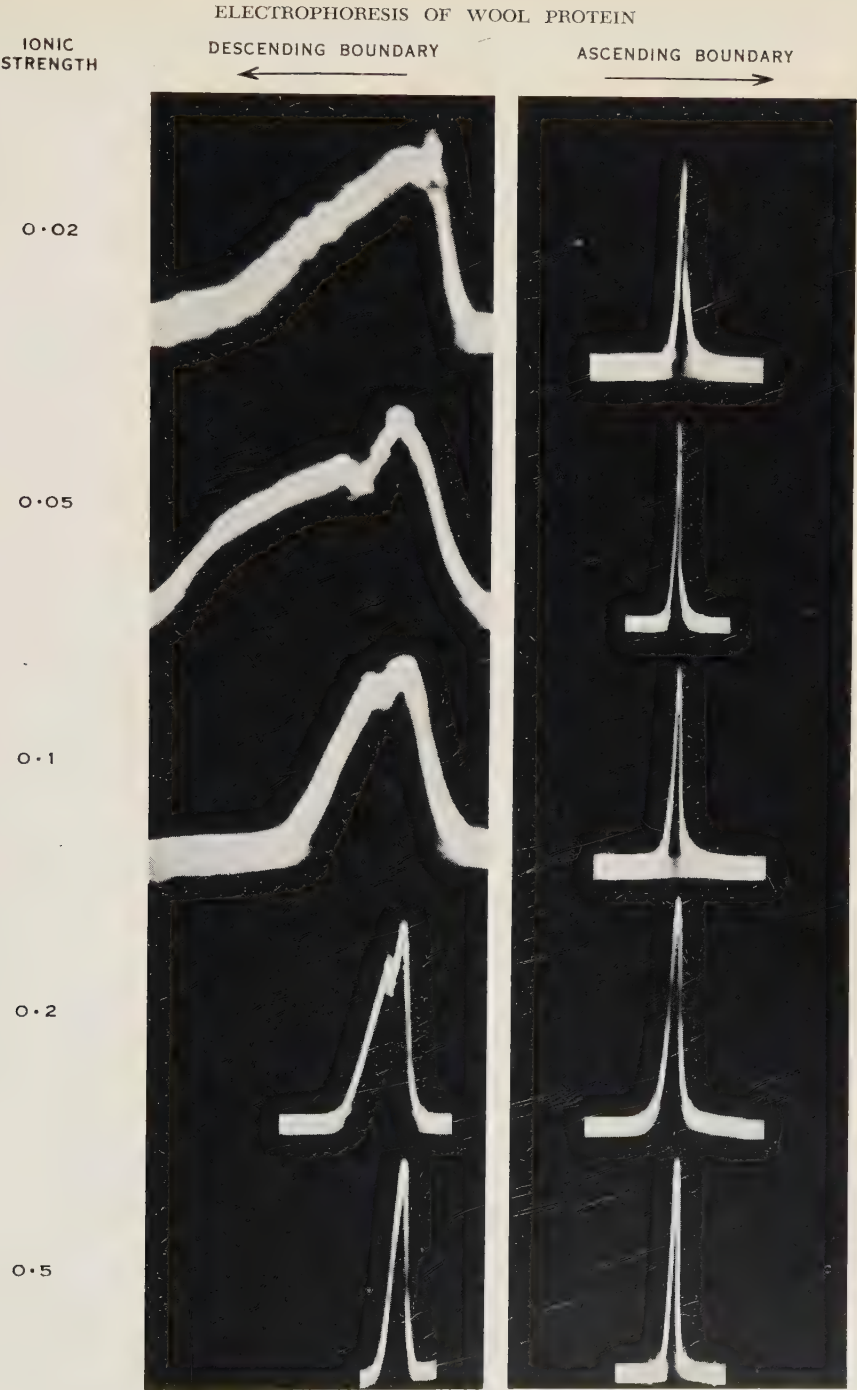
0.6

0.8

1.2

1.8





STUDIES OF THE SPERMICIDAL ACTIVITY OF CHELATING AGENTS

By I. G. WHITE*

[Manuscript received November 15, 1954]

Summary

In studies covering 4-hr periods at room temperature of the effect of 0.1mM chelating agents on the motility of ram, bull, rabbit, and human spermatozoa, the following significant effects were seen:

(i) Cupferron, *o*-phenanthroline, and 1-nitroso-2-naphthol were spermicidal to all four species.

(ii) Sodium diethyldithiocarbamate, ethyl potassium xanthate, 8-hydroxyquinoline, and salicylaldoxime were toxic to bull, rabbit, and human spermatozoa.

(iii) Diphenylthiocarbazone was toxic to rabbit and human spermatozoa and with the latter species phenylthiohydantoic acid and dithio-oxamide were also spermicidal.

In studies of the effect of 0.2mM metals on the toxicity of chelating agents for bull spermatozoa, it was found that:

(iv) The toxicity of sodium diethyldithiocarbamate, 1-nitroso-2-naphthol, and *o*-phenanthroline was reduced by cobalt.

(v) Copper increased the spermicidal activity of 1-nitroso-2-naphthol, cupferron, 8-hydroxyquinoline, and ethyl potassium xanthate. Cadmium had a similar effect on ethyl potassium xanthate. It is suggested that the spermicidal activity of these chelating agents may be dependent on combination with trace concentration of copper and other heavy metals, and that the protective effect of cobalt may be due to it competing to form a non-toxic complex.

I. INTRODUCTION

Chelating agents, i.e. organic molecules that bind metals, forming ring structures, have been used for some time as micro-reagents in analytical chemistry (see Martell and Calvin 1952). Many of them are active in low concentrations at physiological hydrogen ion concentrations (Albert and Gledhill 1947) and hence might be expected to be of value for investigating the trace element requirements of cells.

One such chelator, viz. 8-hydroxyquinoline (oxine) is a powerful antiseptic (see Albert 1944) and fungicide (Rigler and Greathouse 1941) and there is evidence that this may be due, in part at least, to its ability to form complexes with essential trace elements. Thus Zentmyer (1944) found that zinc reduced the fungicidal activity of oxine whilst Albert *et al.* (1947) have shown that cobalt antagonizes its bacteriostatic effect on Gram + organisms, and that iron and zinc have a similar action with Gram - cells. It has also been reported that manganese, cobalt, and iron prevent the inhibitory effect of oxine on glutamic acid assimilation by *Staphylococcus aureus* (Gale 1949).

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More recently McNaught, Owen, and Smith (1950) have studied the effect of chelating agents on rumen bacteria and found that oxine, *aa'*-dipyridyl, and *o*-phenanthroline strongly depress growth. The inhibition produced by oxine could be reversed only partly by metals but that due to the other two chelating agents was completely reversed by iron and partly so by cobalt and zinc. *o*-Phenanthroline has also been found toxic to lactic acid bacteria by MacLeod (1952) who, however, concludes that its action is in large part due to factors not concerned with its ability to bind metals.

Some chelating agents (e.g. oxine and *aa'*-dipyridyl) have also recently been shown to have trypanocidal properties (Ryley 1953).

In this paper, the effect of a number of chelating agents on the motility of ram, bull, rabbit, and human spermatozoa has been studied with a view to determining their trace element requirements.

II. MATERIALS AND METHODS

(a) *Technique and Diluents*

The method of collecting semen and the composition of the diluting fluid were the same as described previously (White 1954).

Semen was diluted 1 in 40 in small tubes for motility observations which were made at room temperature. All tests have been replicated on four ejaculates.

Motility was scored at hourly intervals over a 4-hr period by the system of Emmens (1947). Full motility was rated as 4 and complete immotility as zero, but in presenting results the actual scores have been multiplied by 4, since quarter grades were frequently used.

The chelating agents were B.D.H. laboratory reagents and the metals were added as the A.R. sulphate salts.

(b) *Statistical Analysis*

With the exception of Table 3 (where a simple *t*-test has been used) the results of experiments have been evaluated by the analysis of variance (see Fisher 1946) with isolation of sums of squares attributable to differences between ejaculates and treatments. The total value for the experimental period has been used as unit observation and the treatment-ejaculate interaction mean square as the error term. Differences between ejaculates are often highly significant, so that the accuracy of comparisons is much improved by analysis which both takes this into account and utilizes an overall estimate of error. The standard error of the difference between a pair of treatment means is calculated using the formula

$$S_d = \frac{2s^2}{r},$$

where S_d = standard error of the difference between a pair of treatment means,
 s^2 = the interaction mean square, and
 r = number of replications.

The significance of differences between pairs of treatment means has then in effect been assessed by a *t*-test using S_d and the degrees of freedom associated with s^2 . Where three controls were used for safety, the best estimate (when these controls are homogeneous) of the value of S_d for any one treatment *v.* controls is

$$S_d = \frac{4s^2}{3r}.$$

TABLE 1

EFFECT OF 0.1 mM CHELATING AGENTS ON THE TOTAL MOTILITY SCORE ($\times 4$) OF SPERMATOOA OVER A 4 HR PERIOD. EACH VALUE REPRESENTS THE MEAN OF FOUR EJACULATES

Chelating Agent	Ram	Bull	Rabbit	Human
Nil	72	49	63	53
Nil	69	53	64	57
Nil	72	53	63	58
Cupferron	49**	17**	17**	21**
Titan yellow	73	52	65	53
Quinalizarin	73	52	63	52
Nitroso-R salt	73	49	59	52
Thioglycollic acid	73	55	68	51
Chromotropic acid	73	49	66	52
Ethyl potassium xanthate	68	12**	13**	7**
<i>o</i> -Phenanthroline	34**	15**	12**	32**
Thiourea	72	49	66	46
Phenylthiohydantoic acid	72	52	66	25**
Salicylaldehyde	69	33**	35**	28**
1-Nitroso-2-naphthol	35**	16**	8**	12**
Sodium diethyldithiocarbamate	62	3**	4**	1**
Dithio-oxamide	73	39	65	29*
8-Hydroxyquinoline (Oxine)	61	30**	38**	22**
$\alpha\alpha'$ -Dipyridyl	73	49	63	50
Benzoinoxime	73	48	67	50
Sodium dihydroxytartrate osazone	69	47	53	45
<i>p</i> -Dimethylaminobenzalrhodanine	73	50	66	44
Diphenylcarbazine	73	51	62	50
Mercaptobenzothiazole	64	41	58	
Diphenylthiocarbazon	73	45	38**	25**

** Significantly toxic, $P < 0.01$.

III. RESULTS

(a) Toxicity of Chelating Agents

Table 1 shows the effect on the motility of ram, bull, rabbit, and human spermatozoa of over 20 chelating agents (0.1 mM) that combine with metals at physiological hydrogen ion concentrations (Albert and Gledhill 1947). The results have been subjected to variance analysis with omission of the sodium diethyldithiocarbamate group in the case of bull, rabbit, and human samples.

since it was rapidly toxic to these spermatozoa and the variances are extremely low. Variances of the remaining treatment groups are probably not completely independent of the level of response. Since, however, an effect has only been judged significant when the probability of it being due to chance is less than 1 in 100 this is of little consequence. The summary of the analyses of variance (Table 2) shows significant variation between treatments for each species. The difference between control and experimental groups must be greater than $2.7S_d$ for significance at the 1 per cent. level. Differences between treatment and control means must therefore exceed the following to attain significance: ram—12, bull—12, rabbit—13, human—14.

Three control groups were provided in these tests, but it is clear that they did not differ significantly within any species.

TABLE 2

SUMMARY OF THE ANALYSES OF VARIANCE OF THE DATA IN TABLE 1, SHOWING VARIANCE RATIOS WITH THE ERROR MEAN SQUARE IN ITALICS AT THE BASE OF THE COLUMNS

Source of Variation	Ram		Bull		Rabbit		Human	
	D.F.	V.R.	D.F.	V.R.	D.F.	V.R.	D.F.	V.R.
Between treatments	24	8.3**	23	12.2**	23	22.8**	22	17.6**
Between ejaculates	3	6.2**	3	12.4**	3	26.6**	3	11.5**
Interaction	72	<i>60</i>	69	<i>67</i>	69	<i>73</i>	66	<i>82</i>

** $P < 0.01$.

Cupferron, *o*-phenanthroline, and 1-nitroso-2-naphthol were toxic to the spermatozoa of all four species. Sodium diethyldithiocarbamate, ethyl potassium xanthate, oxine, and salicylaldehyde significantly depressed the motility of bull, rabbit, and human spermatozoa, the effect with the first two chelators being particularly marked. Diphenylthiocarbazon proved toxic to rabbit and human spermatozoa, whilst with the latter species phenylthiohydantoic acid and dithio-oxamide were also spermicidal.

(b) Effect of Heavy Metals on Toxicity of Chelating Agents

If the spermicidal activity of these chelators is due to their depriving spermatozoa of essential trace elements, it should be possible to prevent it by adding the appropriate element to the diluent.

Table 3 shows the effect of adding 0.2 mM manganese, iron, cobalt, copper, zinc, and cadmium to bull spermatozoa in the presence of sodium diethyldithiocarbamate, 1-nitroso-2-naphthol, salicylaldehyde, *o*-phenanthroline, cupferron, oxine, and ethyl potassium xanthate. None of the above metals were themselves toxic to bull spermatozoa at this concentration (White 1955).

The variances of the groups in Table 3 were, by inspection, heterogeneous. Particular interest only attaches to those metals causing either a marked decrease

or increase in the toxicity of the chelating agents. Detailed analysis (by the *t*-test) has only been made, therefore, in the comparison of the cobalt group (D) with (A). Cobalt caused a highly significant decrease in the toxicity of sodium diethyldithiocarbamate ($t = 6.1$, d.f. = 7) and 1-nitroso-2-naphthol ($t = 4.3$, d.f. = 7) and a significant decrease in that of *o*-phenanthroline ($t = 3.3$, d.f. = 7). It is obvious that copper increased the toxicity of 1-nitroso-2-naphthol, cupferron, oxine, and ethyl potassium xanthate and that cadmium also potentiated the latter chelator.

The opposing effects of 0.2 mM copper and 0.2 mM cobalt on the toxicity of 0.1 mM 1-nitroso-2-naphthol were further studied in a factorial experiment (Table 4). Direct factorial analysis is again not possible because of the very low variance of the copper-nitrosodithiol group. In the absence of the chelating agent, however, copper and cobalt were again obviously not toxic. It is also clear that cobalt tended to decrease the toxicity of 1-nitroso-2-naphthol and copper to increase it, whilst cobalt overcame the effect of added copper.

TABLE 3

EFFECT OF 0.2 mM METAL IONS ON THE TOXICITY OF 0.1 mM CHELATING AGENTS FOR BULL SPERMATIZOEA. VALUES REPRESENT THE MEAN TOTAL MOTILITY SCORE ($\times 4$) OVER A 4 HR PERIOD FOR FOUR EJACULATES

Chelator	Control	Chelator and Following Metal:						
		Nil (A)	Manganese (B)	Iron (C)	Cobalt (D)	Copper (E)	Zinc (F)	Cadmium (G)
Sodium diethyldithiocarbamate	64	9	9	13	45**	14	2	5
1-Nitroso-2-naphthol	54	20	18	16	56**	3†	15	14
<i>o</i> -Phenanthroline	68	41	41	29	68*	30	13	11
Salicylaldehyde	63	26	34	18	18	13	24	23
Cupferron	55	18	20	10	17	0†	8	18
8-Hydroxyquinoline	57	21	23	12	17	1†	11	19
Ethyl potassium xanthate	65	30	41	20	34	1†	17	0†

* Significantly better than (A), $P < 0.05$.

** Highly significantly better than (A), $P < 0.01$.

† Obviously more toxic than (A).

Table 3 shows that added copper itself had little effect on the toxicity of sodium diethyldithiocarbamate. However, it reduced the ability of cobalt to make the chelating agent less toxic, as can be seen from the second factorial experiment in Table 4. The situation is clearly complex with obvious interactions between the chelating agent and metals; the data have therefore been split and analysed separately in the presence and absence of sodium diethyldithiocarbamate. The summary of the analysis of variance (Table 5) shows that in the absence of the chelating agent, copper and cobalt are not toxic,

singly or in combination, nor is there any interaction between them. In the presence of the chelating agent cobalt is significantly beneficial, copper has no effect, and there is a significant copper-cobalt interaction. Copper would not

TABLE 4

RESULTS OF FACTORIAL EXPERIMENTS SHOWING THE INTERACTION BETWEEN CHELATING AGENTS (0.1 mM), COBALT (0.2 mM), AND COPPER (0.2 mM). VALUES REPRESENT THE TOTAL MOTILITY SCORE ($\times 4$) OVER A 4 HR PERIOD

Chelating Agent	Ejaculate	Control	Cobalt	Copper	Cobalt + Copper	Chelator	Chelator + Cobalt	Chelator + Copper	Chelator + Cobalt + Copper
1-Nitroso-2-naphthol	1	44	45	44	38	28	49	0	39
	2	68	70	68	67	44	69	0	70
	3	62	62	56	53	41	62	2	49
	4	57	58	56	53	37	59	1	54
	Mean	57	58	56	53	37	59	1	54
Sodium diethyl-dithiocarbamate	1	51	52	45	39	6	28	15	14
	2	48	52	42	50	3	39	18	12
	3	68	72	71	71	1	64	26	16
	4	59	52	53	48	12	39	10	10
	Mean	57	57	50	52	6	43	17	13

be expected to have this effect if the function of added cobalt was merely to make good that bound by chelation.

TABLE 5

SUMMARY OF THE ANALYSES OF VARIANCE OF THE SODIUM DIETHYLDITHIOCARBAMATE DATA IN TABLE 4, SHOWING VARIANCE RATIOS WITH THE INTERACTION MEAN SQUARE IN ITALICS AT THE BASE OF THE COLUMNS

Source of Variation	D.F.	Absence of Chelator	Presence of Chelator
Between treatments:	3	1.6	14.7**
Effect of cobalt	1	0.0	15.3**
Effect of copper	1	4.5	4.5
Cobalt/copper interaction	1	0.1	24.3**
Between ejaculates	3	28.3**	1.4
Interaction	9	17	70

** $P < 0.01$.

The explanation might be that the chelator is merely less toxic in combination with cobalt and that copper displaces cobalt from sodium diethyldithiocarbamate when both metals are present in equal concentrations.

This raises the question as to what is the minimum amount of copper that will antagonize a given amount of cobalt in the presence of sodium diethyl-

dithiocarbamate. Table 6 shows the results of an experiment to investigate this point and Table 7 the summary of the analysis of variance. For an effect signi-

TABLE 6

EFFECT OF INCREASING CONCENTRATIONS OF COPPER ON THE EFFICACY OF COBALT AS AN ANTAGONIST TO SODIUM DIETHYLDITHIOCARBAMATE

Ejaculate	Control	10 ⁻¹ mM Sodium Diethyldithiocarbamate +					
		No Cobalt	2 × 10 ⁻¹ mM Cobalt and Following Copper Concentrations:				
			0	10 ⁻⁴ mM	10 ⁻³ mM	10 ⁻² mM	10 ⁻¹ mM
1	40	8	35	16	31	10	11
2	54	11	30	27	29	11	7
3	66	23	68	68	66	44	28
4	55	21	43	43	48	13	13
Mean	54**	16	44**	39**	44**	20	15

** Highly significantly better than the no cobalt group, $P < 0.01$.

ficant at the 1 per cent. level, treatment means must differ by a minimum of 14. It can be seen that 0.01 mM copper will prevent 0.20 mM cobalt from reducing the toxicity of sodium diethyldithiocarbamate; at copper concentrations below this value, however, cobalt is active.

TABLE 7

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 6 SHOWING VARIANCE RATIOS WITH THE INTERACTION MEAN SQUARE IN ITALICS

Source of Variation	D.F.	V.R.
Between treatments	6	20.5**
Between ejaculates	3	26.9**
Interaction	18	<i>49</i>

** $P < 0.01$.

IV. DISCUSSION

(a) Toxicity of Chelating Agents

No previous systematic studies seem to have been made on the effect of chelating agents on mammalian spermatozoa, although chinosol, which contains oxine, has been used for some time as a chemical contraceptive (Baker 1931). Other chelating agents tried in this experiment were much more spermicidal than oxine (Table 1) and might be useful in this regard. It may be noted

that the efficacy of chinosol and some of the other chelators should be greatly increased by the addition of copper and other metals (Table 3).

Invertebrate spermatozoa apparently differ in their response to chelating agents since Tyler (1953) reports that "Versene," diethyldithiocarbamate, oxine, and cupferron are beneficial to diluted sea-urchin spermatozoa in the concentrations used here.

(b) Effect of Metals on Toxicity of Chelating Agents

It is tempting to interpret the mitigating effect of cobalt on the toxicity of sodium diethyldithiocarbamate, 1-nitroso-2-naphthol, and *o*-phenanthroline to mean that they function by depriving spermatozoa of cobalt.

On the other hand, the fact that copper and cadmium increase the toxicity of some of the chelating agents suggests that their spermicidal activity might be normally dependent on combination with trace concentrations of such metals in the diluent and semen. The reverse effect of cobalt with the above three chelators could be due to it competing to form a non-toxic complex. In those cases where cobalt exerts no protective action against chelating agents it is possible that the stability of the toxic metal complexes is very great relative to that of cobalt.

It may be noted that Mason (1948) has found copper oxinate to be a more powerful fungicide than oxine itself, whilst Anderson and Swaby (1951) report that in the absence of copper or iron, oxine is not fungistatic to *Aspergillus niger* at all. Essentially similar findings have been made by Rubbo, Albert, and Gibson (1950) for Gram + bacteria.

V. ACKNOWLEDGMENTS

I am indebted to Professor C. W. Emmens for his interest and advice, to Mr. A. W. Blackshaw for collecting ram semen, to the University Farm and Camden Park Estate for bull semen, and to the Women's Hospital, Sydney, for the supply of human semen.

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A STUDY OF THE JOINT ACTION OF OESTRONE, OESTRADIOL-3,17 β , AND OESTRIOL

By P. J. CLARINGBOLD*

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Summary

When administered by the intravaginal route oestrone, oestradiol-3,17 β , and oestriol have a mutually antagonistic action on the vaginal response of the ovariectomized mouse. Using the subcutaneous route, these oestrogens are found to have an additive action, behaving simply as dilutions of one active substance.

It is suggested that these results support the view that following systemic administration the natural oestrogens are converted to a common form or mixture in the liver. This common form or mixture elicits responses in the target organs.

The present study confirms the earlier work on the slope and relative position of dose response lines obtained with oestrone, oestradiol-3,17 β , and oestriol using either subcutaneous or intravaginal administration.

I. INTRODUCTION

Several investigations have indicated differences in the mode of action of oestrone, oestradiol-3,17 β , and oestriol on the vaginal epithelium of the ovariectomized mouse. When administered intravaginally, oestrone and oestriol reach equal potency given optimum conditions of administration, while under these conditions oestradiol-3,17 β is 1.5 times as active (Biggers and Claringbold 1954a). The slope of the dose response line depends on the oestrogen used to stimulate the epithelium locally. Oestrone elicits responses fitted by a line twice as steep as the dose response line for oestradiol-3,17 β (Biggers and Claringbold 1953). In this work it was also demonstrated that the thyroid status influences responses to oestrone but not to oestradiol-3,17 β . Claringbold (1953) found that while cyanide increased the percentage response to oestrone it was without effect on the response to oestradiol-3,17 β .

It was thought that studies of the joint or simultaneous action of the three oestrogens would help to elucidate their mode of action. While Bliss (1939) first discussed some of the aspects of the study of the joint action of related compounds these ideas have been extended by Finney (1952) and Plackett and Hewlett (1951). Recently Claringbold (1954) suggested a new approach to the problem based on a special experimental design and in that paper details of the design and methods of analysis are fully given. Only a brief outline of the statistical methods will be given here.

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II. MATERIALS AND METHODS

A colony of 400 albino ovariectomized mice bred in this Department were used. Before their use in tests the mice were "primed" by the subcutaneous injection of $1\text{ }\mu\text{g}$ of oestrone in peanut oil. Intravaginal injections were made in 1 per cent. aqueous egg albumin (Biggers 1953) while subcutaneous injec-

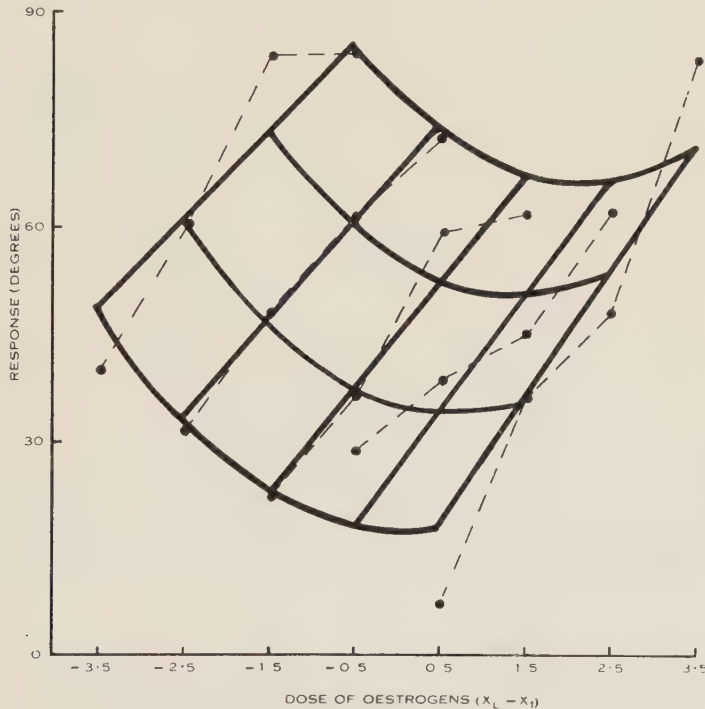


Fig. 1.—The following regression equation has been computed using the data of Table 1:

$$Y = 45.1 + 15.1 X_L + 5.6 X_1 + 2.7 X_1^2 - 1.4 X_1 X_L,$$

where Y = angle response, and X_L is the partition. The equation has been plotted using the difference $(X_L - X_1)$ of the independent variables as abscissa in order to reduce a three-dimensional relationship to a two-dimensional one. This places the dose response line obtained with oestradiol-3,17 β alone to the extreme left and that with oestrone alone on the extreme right. The experimental points are indicated joined by thin, broken lines. The calculated surface is indicated in thick lines.

tions were made in peanut oil (Emmens 1950). For either route of administration, two injections were given 24 hr apart. The oestrone, oestradiol-3,17 β , and oestriol were obtained from Organon Laboratories.

In tests involving intravaginal administration, vaginal smears were taken at 48, 56, and 72 hr after the first applications. With subcutaneous administration smears were taken 56, 72, and 80 hr after the first injection. Response in each animal was scored as positive if at least one vaginal smear contained cornified or nucleated cells or both in the absence of leucocytes.

III. DESIGN AND RESULTS OF EXPERIMENTS

(a) Joint Action of the Natural Oestrogens Administered Intravaginally

The design of the first experiment involving the two oestrogens, oestrone, and oestradiol-3,17 β , is indicated in Table 1. Four levels of total or joint dose entered the design, i.e. either 1, 2, 4, or 8×10^{-4} μ g of a mixture of oestrone and

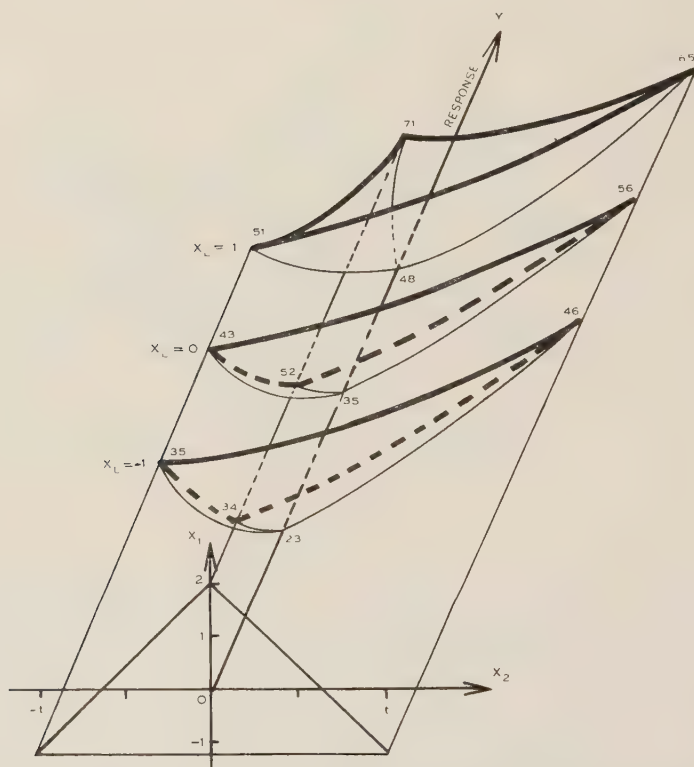


Fig. 2.—Representation of the regression surface estimated in the analysis summarized in Table 3. The two-dimensional partitioning of the dose governed by X_1 and X_2 is indicated in the base triangle. The dose response lines obtained with either oestrone, oestradiol-3,17 β , or oestriol by themselves lie above the back, right-hand, and left-hand corners of this triangle. Response is plotted above the triangle, as on the sides of an equilateral triangular prism. Three levels of response corresponding to the three values of joint dose are plotted. The replication factor is ignored in this figure. The response coordinate (Y) runs through the centre of the three surfaces and the triangle and corresponds to a 0.33, 0.33, 0.33 mixture of the three oestrogens. The small figures at the corners and centre of the surfaces indicate the expected response at these points on the basis of the estimated equation (ignoring the replication coefficient).

oestradiol-3,17 β was administered. At each level of joint dose, five different partitionings of this dose were made between the two oestrogens. These partitionings are in a linear order and were coded as indicated in Table 1.

TABLE 1

PERCENTAGE RESPONSE OF GROUPS OF 18 OVARECTOMIZED MICE TO 20 DIFFERENT DOSES OF BOTH OESTRONE AND OESTRADIOL-3,17 β

In order to save space the partitioning of the lowest joint dose is indicated in full. This set was coded $X_L = -1.5$. Successive doses are obtained by repeated doubling of these doses and correspond to the cases where $X_L = -0.5, 0.5$, and 1.5 . Thus when $X_L = -0.5$ and $X_1 = 0$, 1×10^{-4} μ g of both oestrone and oestradiol-3,17 β were administered

Joint Dose		Response (%)				
Dose Oestrone (10^{-4} μ g)	Dose Oestradiol-3,17 β (10^{-4} μ g)	X_1	X_L			
			-1.5	-0.5	0.5	1.5
1.00	0.00	-2	0	33	55	100
0.75	0.25	-1	28	42	50	78
0.50	0.50	0	11	33	72	70
0.25	0.75	1	28	55	78	89
0.00	1.00	2	49	78	100	100

Analysis of variance (Table 2) of the percentage data following the empirical angular transformation (Claringbold, Biggers, and Emmens 1953) indicates that the five dose response lines are linear. It also indicates that mixtures of the two oestrogens are considerably less potent than predicted on the basis of additivity of the activities of the components. The difference in slope be-

TABLE 2

ANALYSIS OF VARIANCE OF THE DATA OF TABLE 1 EMPLOYING THE EMPIRICAL ANGULAR TRANSFORMATION (CLARINGBOLD, BIGGERS, AND EMMENS 1953)

Source of Variation	D.F.	Mean Square	F
Doses (X_L)	(3)		
Linear	1	5730	118.1***
Quadratic	1	48	1.0
Cubic	1	1	<1
Partition (X_1)	(4)		
Linear	1	1232	25.4***
Quadratic	1	402	8.3**
Cubic	1	17	<1
Quartic	1	46	<1
Doses \times partition	(12)		
Linear \times linear	1	98.0	2.0
Remainder	11	76.4	1.6
Theoretical variance	∞	48.5	

** $0.01 > P > 0.001$.

*** $P < 0.001$.

tween the dose response line obtained with oestrone and that obtained with oestradiol-3,17 β is of the same order as that reported by Biggers and Claringbold (1953), but fails to reach significance owing to the small size and nature of the design of the experiment. As a result the interaction term has been included in the graphical representation of the experimental results in Figure 1. It would appear that the two oestrogens exhibit a mutually antagonistic action.

TABLE 3

PERCENTAGE RESPONSE OF GROUPS OF 12 OVARIECTOMIZED MICE TO DIFFERENT COMBINATIONS OF DOSES OF OESTRONE, OESTRADIOL-3,17 β , AND OESTRIOL

The partitioning at the lowest joint dose ($X_L = -1$) is shown in full in each replicate. Subsequent levels of joint dose are obtained by repeated doubling and correspond to $X_L = 0$ and 1

Joint Dose				Coordinates		Response (%)		
	Dose Oestrone (10 ⁻⁴ μ g)	Dose Oestradiol-3,17 β (10 ⁻⁴ μ g)	Dose Oestriol (10 ⁻⁴ μ g)	X_1	X_2	$X_L =$		
						-1	0	1
First replicate $X_R = -1$	1.00	0.00	0.00	2	0	17	42	83
	0.67	0.33	0.00	1	$t/3^*$	0	33	75
	0.33	0.67	0.00	0	$2t/3$	33	33	75
	0.00	1.00	0.00	-1	t	58	58	100
	0.00	0.67	0.33	-1	$t/3$	17	33	67
	0.00	0.33	0.67	-1	$-t/3$	33	33	58
	0.00	0.00	1.00	-1	$-t$	25	50	42
	0.33	0.00	0.67	0	$-2t/3$	25	42	42
	0.67	0.00	0.33	1	$-t/3$	0	25	75
	0.33	0.33	0.33	0	0	17	25	58
Second replicate $X_R = 1$	1.00	0.00	0.00	2	0	42	50	75
	0.50	0.50	0.00	1/2	$t/2$	17	33	83
	0.00	1.00	0.00	-1	t	75	67	83
	0.00	0.50	0.50	-1	0	33	42	67
	0.00	0.00	1.00	-1	$-t$	50	42	100
	0.50	0.00	0.50	1/2	$-t/2$	17	42	58
	0.67	0.17	0.17	1	0	33	33	58
	0.17	0.67	0.17	-1/2	$t/2$	50	50	58
	0.17	0.17	0.67	-1/2	$-t/2$	33	33	50
	0.33	0.33	0.33	0	0	17	42	42

* $t = \sqrt{3}$.

A further experiment was carried out employing oestrone, oestradiol-3,17 β , and oestriol. The experimental design employed in this work was specially devised for this study and has been described fully elsewhere (Claringbold 1954). Three levels of joint dose were chosen for study, namely 0.75, 1.5, and 3×10^{-4} μ g of mixtures of the three oestrogens. The experiment was car-

ried out in two replicates one week apart. In each replicate 10 different methods of partitioning the joint dose amongst the three oestrogens were chosen. These are given in Table 3 together with the mathematical coding of the replicates, levels, and partitions. Two coordinates are required to describe the partitionings since the three doses of the oestrogens at each point sum up to a constant joint dose (within each level).

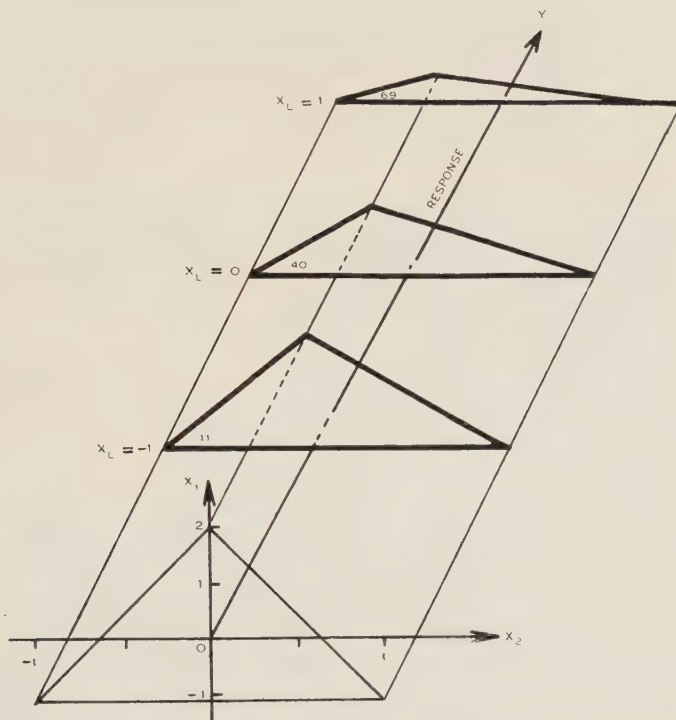


Fig. 3.—Representation of the response surface estimated in the analysis summarized in Table 6. It is plotted in an analogous manner to Figure 2. Response is linearly related to the logarithm of the joint dose (X_L) and its internal coordinates ($X_1 X_2$). The three levels are thus parallel, equidistant, horizontal cuts in the triangular prism resting on the base triangle. The estimated response at each level (ignoring all regression coefficients except the mean and that on X_L) is shown in small figures.

The empirical angular response has been related by a regression analysis to 10 functions of the coordinates of the design (Table 4). This analysis indicates that the oestrogens have a mutually antagonistic action. It also confirms the earlier work indicating that oestradiol-3,17 β is more potent and with oestriol elicits responses fitted by a flatter dose response line than oestrone. The response surface is illustrated in Figure 2.

(b) Joint Action of the Natural Oestrogens Administered Subcutaneously

The design of the experiment in this case is complicated by the fact that the three oestrogens studied have markedly different activities when adminis-

tered by this route. In this more general case a mathematical approach is as follows. Suppose θ_1 , θ_2 , and θ_3 are approximately equivalent doses of oestrone, oestradiol-3,17 β , and oestriol. Then a joint dose (D) may be defined

$$D = a_1 \theta_1 + a_2 \theta_2 + a_3 \theta_3, 0 \leq a_1, a_2, a_3 \leq 1, \quad \dots \quad (1)$$

where

$$a_1 + a_2 + a_3 = 1.$$

Since we are interested in the relationship of angle response to log dose this equation is an unsuitable form since $\log D$ is not a linear function of the multipliers a_1 , a_2 , and a_3 . By means of transformations (Claringbold 1954) equation (1) may be written

$$\log D = \beta_1 \log \theta_1 + \beta_2 \log \theta_2 + \beta_3 \log \theta_3, 0 \leq \beta_1, \beta_2, \beta_3 \leq 1,$$

where

$$\beta_1 + \beta_2 + \beta_3 = 1.$$

The transformations allow sets of a to be determined corresponding to sets of β , and are derived in short below. Briefly, equation (1) may be written

$$D = p \{ p^1 \theta_1 + (1 - p^1) \theta_2 \} + (1 - p) \theta_3, 0 \leq p, p^1 \leq 1$$

$$= p b + (1 - p) \theta_3,$$

$$\log D = q q^1 \log \theta_1 + q (1 - q^1) \log \theta_2 + (1 - q) \log \theta_3$$

$$= \beta_1 \log \theta_1 + \beta_2 \log \theta_2 + \beta_3 \log \theta_3,$$

where

$$p^1 = (r_1^{(1-q^1)} - r_1) / (1 - r_1); \quad r_1 = a_2/a_1,$$

and

$$p = (r_2^{(1-q)} - r_2) / (1 - r_2); \quad r_2 = a_3/b.$$

The experimental design adopted in this study was identical to the one used in the previous experiment, the doses administered being determined by the above transformation. The experiment likewise was in two replicates one week apart. In the first replicate many responses near 0 and 100 per cent. were observed and in the second replicate the logarithmic interval was reduced. The doses administered and their coding is indicated in Table 5, together with the experimental results. The regression analysis is given in Table 6. This analysis indicates that all partitionings at the one level and in the same replicate are equivalent in their effect on percentage response. There is no evidence of any antagonistic action for this route of administration (compare Fig. 3 with Fig. 2), and response is linearly related to the logarithm of the dose of each oestrogen separately or in mixtures.

IV. DISCUSSION

Comparison of the median effective dose (M.E.D.) obtained by the subcutaneous administration of a true oestrogen with the M.E.D. obtained by intravaginal administration of the same oestrogen indicates that less than 1 per cent. of the dose administered subcutaneously finally reaches the vaginal epithelium to initiate a response. Presumably following subcutaneous administration the oestrogen is absorbed from the subcutaneous depot and transported via the blood stream to the target organs. The factors underlying the high systemic/local M.E.D. ratio may be divided into two groups: (i) factors governing the amount of oestrogen utilized or held in other target organs, and (ii)

factors governing the rapid destruction and excretion of oestrogens. The amount of oestrogen utilized in the other target organs such as the uterus and mammary gland is unknown, but since these organs are considerably larger than the vaginal epithelium it would be expected that each would utilize more oestrogen than the vaginal epithelium. Studies with radioactive compounds related to the oestrogens (Twombly 1951) have indicated that oestrogens are rapidly excreted in the bile; up to 80 per cent. of products of the injected oestrogen are found in the bile 5-6 hr after administration. An enterohepatic circulation of oestrogen is set up which probably maintains the level of circulating oestrogen.

TABLE 4

REGRESSION ANALYSIS OF EMPIRICAL ANGULAR RESPONSE ON 10 FUNCTIONS OF THE COORDINATES OF THE DESIGN

Coordinate Function	Regression Coefficient	$t_{(\infty)}$
Constant	35.39 ± 1.81	
X_R	2.61 ± 1.12	2.3*
X_1	1.93 ± 1.78	<1
X_2	2.84 ± 1.69	1.7
X_L	12.30 ± 1.37	9***
$X_1 X_2$	-0.90 ± 1.96	<1
$X_1 X_L$	3.12 ± 1.41	2.21*
$X_2 X_L$	0.54 ± 1.41	<1
X_1^2	3.20 ± 1.32	2.42**
X_2^2	4.21 ± 1.32	3.19**

Deviations from regression: $\chi^2_{(50)} = 49.7$

* $0.05 > P > 0.01$.

** $0.01 > P > 0.001$.

*** $P < 0.001$.

Studies of the optimum conditions of administration of oestrogens (Biggers and Claringbold 1954a) have indicated that an effective dose of oestrogen must be in contact with the vaginal epithelium for 36-48 hr in order to elicit a response. The finding is supported by studies of Biggers and Claringbold (1954b) who found that the number of mitoses in the vaginal epithelium arrested by colchicine was greatly increased in the period. The work supports the view that the level of circulating oestrogen remains raised for 36-48 hr after a subcutaneous injection and the view that, although subcutaneously administered oestrogen rapidly appears in the bile, it is still available to the target organs via the blood stream in a modified form. Biggers and Claringbold (1954b) found that following one maximal dose of oestrone given intravaginally, mitotic activity did not begin to increase until 18 hr had passed. Thus if most oestrogen is lost in the bile in 5-6 hr and the circulating oestrogen practically

disappears, it would be expected that mitosis would begin immediately following injection of oestrogen rather than after a delay. The work suggested that it is the oestrogen from the enterohepatic circulation which finally acts on the

TABLE 5

PERCENTAGE RESPONSE OF GROUPS OF 12 OVARECTOMIZED MICE TO DIFFERENT COMBINATIONS OF DOSES OF OESTRONE, OESTRADIOL-3,17 β , AND OESTRIOL

The partitioning of the middle joint dose ($X_L = 0$) is shown in full for each replicate. In the first replicate the other levels of X_L are -1 and 1 , and the corresponding doses are obtained by respective division and multiplication by 2. The other levels of X_L in the second replicate are $X_L = -0.49$ and 0.49 . The appropriate doses are obtained by successive division and multiplication by 1.4

	Joint Dose			Coordinates		Response (%)		
	Dose Oestrone (10^{-2} μ g)	Dose Oestradiol-3,17 β (10^{-2} μ g)	Dose Oestriol (10^{-2} μ g)	X_1	X_2	$X_L =$		
						-1	0	1
First replicate $X_R = -1$	8.00	0.00	0.00	2	0	8	58	92
	4.70	1.64	0.00	1	$t/3^*$	8	17	83
	2.08	2.96	0.00	0	$2t/3$	0	50	92
	0.00	4.00	0.00	-1	t	8	67	75
	0.00	3.34	3.92	-1	$t/3$	0	58	100
	0.00	2.16	11.06	-1	$-t/3$	25	33	92
	0.00	0.00	24.00	-1	$-t$	8	50	83
	3.68	0.00	12.96	0	$-2t/3$	8	50	83
	6.22	0.00	5.30	1	$-t/3$	25	42	92
	2.68	1.90	4.60	0	0	8	75	100
						-0.49	0	0.49
Second replicate $X_R = 1$	8.00	0.00	0.00	2	0	8	33	67
	3.31	2.34	0.00	$1/2$	$t/2$	17	42	75
	0.00	4.00	0.00	-1	t	8	33	58
	0.00	2.84	6.96	-1	0	17	25	83
	0.00	0.00	24.00	-1	$-t$	8	25	42
	5.07	0.00	8.78	$1/2$	$-t/2$	0	33	50
	3.82	0.66	6.19	1	0	8	42	67
	1.11	3.14	1.82	$-1/2$	$t/2$	8	33	83
	1.66	1.17	12.00	$-1/2$	$-t/2$	17	42	67
	2.68	1.90	4.61	0	0	17	25	58

* $t = \sqrt{3}$.

target organs. Unfortunately, in the mouse, little is known of the constituents of the blood and urinary oestrogens and the nature of the circulating oestrogen cannot be stated or deduced.*

* *Note added in Proof.*—In a recent publication (Stimmel 1955) the metabolism of oestradiol-16- 14 C and oestrone-16- 14 C in dogs has been studied. Following subcutaneous

The present work has indicated differences between the subcutaneous and intravaginal actions of natural oestrogens. Using the subcutaneous route these oestrogens behave simply as dilutions of oestradiol-3,17 β , the most potent under these conditions. All dose response lines of mixtures had similar slopes and the effect of joint dose was equal to that predicted on the basis of additivity of its component oestrogens. With the intravaginal route, however, the findings are completely reversed. The oestrogens are mutually antagonistic in their action and the dose response slopes depend on the oestrogen studied.

TABLE 6
REGRESSION ANALYSIS OF THE DATA OF TABLE 5

Coordinate Function	Regression Coefficient	$t_{(\infty)}$
Constant	40.25 ± 1.12	
X_R	-4.38 ± 1.12	3.9***
X_1	-0.05 ± 1.15	<1
X_2	0.72 ± 1.15	<1
X_L (\log_{10} dose)	97 ± 6	17***

Deviations from regression: $\chi^2_{(55)} = 43.5$

*** $P < 0.001$.

If it is assumed that under the same conditions of administration the slope of the dose response line is a characteristic feature of the response of the vaginal epithelium to different oestrogens, it follows that the oestrogen or mixture of oestrogens finally reaching the vaginal epithelium after subcutaneous administration does not depend on the natural oestrogen administered subcutaneously. In particular the dose response line obtained by the intravaginal administration of oestradiol-3,17 β is the flattest of all natural oestrogens, being roughly one-half of the slope obtained with oestrone. If, following subcutaneous administration of oestrone or oestradiol-3,17 β , only the oestrogen administered finally reached the vaginal epithelium, different dose response slopes are expected and joint doses would have an antagonistic action. This is not the case, the simplest explanation being that the oestrogens are converted by the liver to a common form or mixture which is either excreted in the bile or passes through the blood stream to the target organs.

Following the administration of an effective dose of true oestrogen by the intravaginal route, it acts directly on the cells concerned (Robson and Adler 1940; Emmens 1942; Hardy, Biggers, and Claringbold 1953). The possibility that conversion to oestradiol-3,17 β takes place in the epithelium was suggested

administration of either oestrogen the label is found in the urine as a mixture of oestrone, oestradiol-3,17 β , and oestriol in approximately equal quantities. This is strong evidence that the circulating oestrogen is a mixture derived by metabolism of injected oestrogen as suggested above.

by Biggers and Claringbold (1953). The antagonistic action of the natural oestrogens could be explained in many ways. If interconversions, partial or full, take place, the administration of the final products of this conversion together with the stimulating oestrogen would tend to reduce the amount of final product formed by a mass action effect. Alternatively some process of differential transport or differential utilization may be suggested. The question cannot be fully answered on the basis of present knowledge although the present study strongly indicates that oestrogens undergo changes in the vaginal epithelium.

V. ACKNOWLEDGMENTS

I wish to thank Professor C. W. Emmens and Dr. J. D. Biggers for helpful criticism and advice during the course of this investigation which was supported by grants from the Wool Industry Fund and the Commonwealth Bank of Australia.

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THE SIMULTANEOUS ADMINISTRATION OF OESTRONE BY THE SUBCUTANEOUS AND INTRAVAGINAL ROUTES

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Summary

A mathematical model has been developed for the study of the joint action of a substance administered simultaneously by two routes. The model has been used for the study of the joint action of oestrone on the vagina of the ovariectomized mouse when it is injected simultaneously by the intravaginal and subcutaneous routes.

It has been shown that the intravaginal injection of 95-98 per cent. of an effective dose together with the subcutaneous injection of the remaining 2-5 per cent. results in maximum response.

The significance of these findings to the mode of action of oestrogens is discussed.

I. INTRODUCTION

Several investigations have indicated differences in the subcutaneous and intravaginal actions of oestrogens on the vagina. The first difference to be noted was the much lower median effective dose (M.E.D.) with the intravaginal route of administration when compared with the subcutaneous route (Lyons and Templeton 1936; Mühlbock 1940; Emmens 1941). The next difference noted was the relatively low slope of the dose response line for intravaginal administration when compared with the slope obtained with subcutaneous administration (Emmens 1950). This has since been confirmed by Biggers (1953*a*). Also it has been shown that differences exist in the slope of the dose response lines obtained with the intravaginal administration of oestrone, oestradiol-3,17 β , and oestriol while no differences can be detected with the subcutaneous method (Biggers and Claringbold 1953, 1954; Claringbold 1953).

In completely separate studies, no evidence of a correlation between the response of individuals to the subcutaneous and intravaginal routes of administration has been found (Biggers, Claringbold, and Emmens 1954; Biggers and Claringbold 1955*a*). Further, in other studies with inbred lines, the relative sensitivities of the lines studied depended on the route of administration (Claringbold and Biggers 1955).

It seems that important differences exist in the mode of action of oestrogens given by these two routes, and in order to gain further information on this question studies have been made with oestrone administered simultaneously by the two techniques.

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II. MATERIAL AND METHODS

A colony of about 500 albino mice was used in this work. After ovariectomy the mice were "primed" by a single subcutaneous injection of $1\text{ }\mu\text{g}$ oestrone in peanut oil. The animals were kept in boxes, six animals per box, and these were allotted at random to the treatment groups.

Oestrone was administered subcutaneously in peanut oil, the total dose being given in two injections 24 hr apart, and was given intravaginally in 1 per cent. aqueous egg albumin in two injections simultaneously with the subcutaneous injections (Biggers 1953*b*; Biggers and Claringbold 1954).

Vaginal smears were taken at 48, 56, 72, and 80 hr after the first injections. The animals were scored as positive if at least one smear contained nucleated or cornified cells or both, and no leucocytes.

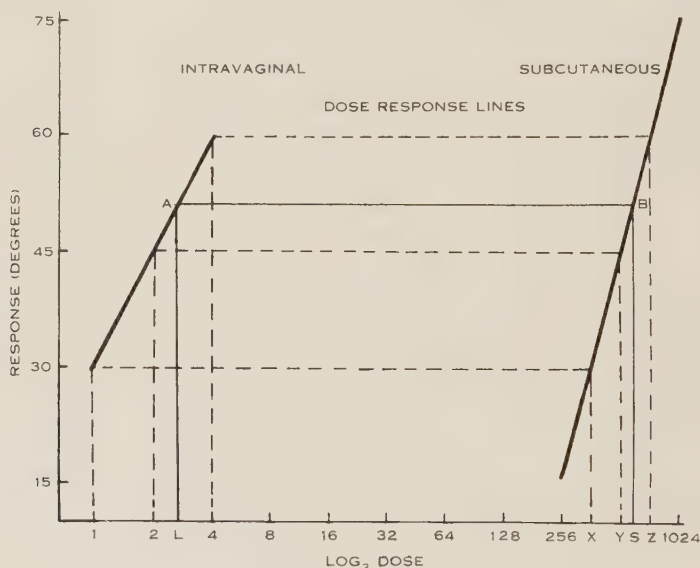


Fig. 1.—Dose response lines on which this study is based.

(a) *Mathematical Model*

The problem is illustrated in Figure 1. The dose response lines obtained by (i) the intravaginal administration of oestrone dissolved in 1 per cent. aqueous egg albumin, and (ii) by the subcutaneous administration of oestrone in peanut oil, are shown. This picture is not invariant since both curves undergo secular variation in both slope and median effective dose (M.E.D.) (see Biggers 1953*a* for a discussion of this effect). The parameters are also dependent on other factors such as the strain of animal used in the test, the technique of administration, and a random component. Broadly, the slope of the dose response line obtained by subcutaneous administration of oestrone is twice as steep as the slope of the dose response line obtained with intravaginal administration. The M.E.D. ratio, an appropriate statistic when use of relative potency is invalidated by non-parallel dose response lines, is about $1/256$.

For each route of administration there is a dose which will cause some arbitrary percentage of animals to respond. Pairs of doses, one for each route, which cause the same arbitrary percentage of animals to respond may be called equivalent doses. Owing to the variations in position of the dose response lines involving roughly factors of 2 for both slope and M.E.D., only approximately equivalent doses may be defined. For any level of response, however, a horizontal line AB may be drawn, and approximately equivalent doses are found at the foot of the perpendiculars to the abscissa, i.e. AL and BS . The general dose given intravaginally is called L and that given subcutaneously is called S .

In the figure the abscissa is shown as a log dose scale with the unit intervals denoted at the actual dose levels. Equally spaced log doses are in general given by a geometric progression. Thus we may define a geometric progression of doses for study, say $1, 2, 4 \times 10^{-4} \mu\text{g}$, for the intravaginal route. Approximately equivalent doses may be obtained by the dotted line construction in Figure 1. In this instance the equivalent doses are $X = 256\sqrt{2}$, $Y = 512$, and $Z = 512\sqrt{2}$. The constants governing these geometric progressions must be defined in advance of the experiment and on these are based the method of studying simultaneous action of both routes.

In practice, an experiment could fail completely because of bad estimation of equivalent doses, or because of uncontrolled shifts in the general level of response. With animals kept under as rigid a set of conditions as possible and all possible care of technique, large shifts are fortunately rare and did not occur in the course of the present work.

Suppose $L \mu\text{g}$ of oestrone is required to produce a given response when administered intravaginally and $S \mu\text{g}$ of oestrone is required to produce approximately the same response when administered subcutaneously. A joint dose (D) may be defined

$$D = aL + (1-a)S, \quad \dots \quad (1)$$

where

$$0 \leq a \leq 1.$$

Thus when $a = 1$ all the dose is given intravaginally and when $a = 0$ all is given subcutaneously.

For both the intravaginal and the subcutaneous routes of administration, the response (expressed as probits, angles, etc.) has been found to be linearly related to log dose. If logarithms are taken in equation (1) it is found that a is not linearly related to log D and is thus inconvenient in analysis. The more useful form of (1) is given by

$$\log D = \beta \log L + (1-\beta) \log S, \quad \dots \quad (2)$$

where

$$0 \leq \beta \leq 1.$$

Thus a in terms of the transformate β is given by

$$a = (A^{1-\beta} - A)/(1-A), \quad \dots \quad (3)$$

where

$$A = S/L.$$

In general approximately equivalent doses administered by each route are given by appropriate geometric progressions. Thus

$$L = a_L r_L \eta; \quad S = a_S r_S \eta, \quad \dots \quad (4)$$

where a_L , a_S , r_L , r_S are constants, and η an equally spaced variable representing each equivalent level of joint administration. Substitution of (4) in (2) yields

$$\log D = \beta \log a_L/a_S + \beta \eta \log r_L/r_S + \log a_S + \eta \log r_S. \quad (5)$$

This is a bilinear function, i.e. linear in β if η is held constant, and linear in η if β is held constant. Selected values of η and β and their combinations, together with the constants defined in (4) selected on the basis of past experience, form an experimental design.

In the analysis, linear transformations of η and β , denoted X_1 and X_2 , form the basic coordinates. A transformate of response (in this case angles) may be related to these coordinates and various coordinate functions derived from them, by the method of regression analysis or analysis of variance (Claringbold, Biggers, and Emmens 1953; Claringbold 1955a).

(b) Special Orthogonal Comparisons

A common method of describing response curves is by the use of orthogonal polynomial coefficients in the analysis of variance (Fisher and Yates 1953). This method, however, provides the simplest description of the data and does not test specific hypotheses. Loraine (1952) has derived a special set of orthogonal coefficients which test for departures from linearity as successive points of the response line are included in the analysis. In this method the first comparison establishes the linearity of the first three points and the second comparison tests the hypothesis that the fourth point is collinear with the first three, and so on.

In the present work we are interested in departures from linearity of the relationship of response to β , in the interval between the extreme values of β . Also it is of interest to test whether equivalent doses have been given. Five levels of β have been used in both experiments. A set of comparisons has been derived for these purposes, and is shown for the case $n=5$, as follows:

Name	k_1	k_2	k_3	k_4	k_5	Sk_i^2
A	-1	0	0	0	1	2
M	1	0	-2	0	1	6
N	2	-3	2	-3	2	30
E	0	-1	0	1	0	2

A fuller set for other values of n has been given by Claringbold (1955b). For convenience each comparison is denoted by the Greek capital letters A to E inclusive.

The comparison A tests whether the mean response to extreme values of β are different. The second comparison, M , tests whether the response to the mid-value of β is collinear with the response to the extreme values. The third comparison, N , tests for a difference between the mean response to the first, third, and fifth values of β and the mean response to the second and fourth values. The fourth comparison, E , tests for a difference between the response to the second and fourth values of β .

In the subsequent analyses, orthogonal polynomial coefficients have been used to examine the relationship of response to dose level (η). The special coefficients have been used to study the relationship with β and also to derive

TABLE 1

PERCENTAGE RESPONSE OF MICE TO THE JOINT ADMINISTRATION OF OESTRONE BY BOTH THE INTRAVAGINAL AND SUBCUTANEOUS ROUTES OF ADMINISTRATION (EXPT. 1, 18 ANIMALS PER GROUP)

Joint Dose of Oestrone		Logarithmic Coding		Response (%)	
Intravaginal Route (10^{-4} μ g)	Subcutaneous Route (10^{-2} μ g)	X_1	X_2	Day 1	Day 2
0	4.10	-1.5	-2	11	22
0	5.80	-0.5	-2	22	39
0	8.10	0.5	-2	39	39
0	11.40	1.5	-2	83	78
0.76	0.98	-1.5	-1	17	17
1.48	1.51	-0.5	-1	44	44
2.86	2.31	0.5	-1	44	67
5.53	3.55	1.5	-1	94	83
0.94	0.25	-1.5	0	11	28
1.87	0.37	-0.5	0	56	22
3.73	0.52	0.5	0	39	50
7.21	1.05	1.5	0	89	83
0.99	0.04	-1.5	1	11	33
1.97	0.08	-0.5	1	44	61
3.93	0.15	0.5	1	83	83
7.82	0.25	1.5	1	100	100
1.00	0	-1.5	2	11	22
2.00	0	-0.5	2	39	44
4.00	0	0.5	2	78	78
8.00	0	1.5	2	67	72

Constants

$$a_L = 2.8 \times 10^{-4}$$

$$r_L = 2$$

$$a_S = 6.9 \times 10^{-2}$$

$$r_S = \sqrt{2}$$

$$\eta = 1.5, -0.5, 0.5, 1.5$$

$$\beta = 0, 0.25, 0.5, 0.75, 1.0$$

the coefficients for the interactions between η and β , i.e. departure from parallelism of the dose response lines at different values of β .

The design, results, and analysis of each experiment will be described in turn.

III. RESULTS

Two experiments, each of two replicates a week apart, have been carried out. In the first the whole range of a has been explored while in the second a restricted region only has been subjected to closer study.

(a) *Experiment 1.* $0 \leq a \leq 1$, i.e. $0 \leq \beta \leq 1$. (Table 1)

Five levels of β were used in this experiment: 0, 0.25, 0.50, 0.75, 1.00, and were transformed to -2, -1, 0, 1, 2 for analysis. The corresponding values of a were obtained using equations (4) and (3) and the constants shown at the foot of Table 1. The doses administered for the four values of η are shown in Table 1 together with the results obtained in two replicates.

TABLE 2
ANALYSIS OF VARIANCE OF THE DATA OF TABLE 1

Source of Variation	D.F.	Mean Square	F
Days	1	70	1.4
Doses (X_1)	(3)		
Linear	1	10239	211***
Quadratic	1	7	<1
Cubic	1	47	<1
Partition (X_2)	(4)		
A	1	127	2.6
M	1	2	<1
N	1	608	12.5***
E	1	342	7.1**
First order interactions:			
Days \times doses	3	71	1.5
Days \times partition	4	19	<1
Doses \times partition	(12)		
Linear \times A	1	2	<1
Linear \times M	1	6	<1
Linear \times N	1	198	4.1*
Linear \times E	1	80	1.6
Remainder	8	94	1.9
Other interactions	12	35	<1
Theoretical variance	∞	48.5	

* $0.05 > P > 0.01$.

** $0.01 > P > 0.001$.

*** $P < 0.001$.

An analysis of variance has been computed with the empirical angular transformation. This method was used in view of the large number of observations made, since in this situation the solution closely approaches the maximum likelihood solution (see Claringbold, Biggers, and Emmens 1953). It is necessary to discuss the dose (η) and partition (β) effects separately in detail.

(i) *Dose* (η).—The analysis of variance (Table 2) indicates that the dose response lines may be considered linear. The analysis of the dose \times partition interactions shows that the mean slope at the second and fourth values of β is greater than at other values of β . Examination of the slopes of the individual dose response lines shows that this effect is largely due to increased slope at $\beta = 0.75$. For each value of β the logarithmic interval in any base (ϕ) between successive joint doses is given, in general by

$$\begin{aligned}\log_{\phi} I_{\beta} &= \log_{\phi}(r_L^{\beta} \times r_S^{(1-\beta)}) \\ &= \beta \log_{\phi} r_L/r_S + \log_{\phi} r_S,\end{aligned}$$

where I_{β} is the ratio of successive joint doses for a fixed β . In the present case

$$\begin{aligned}\log_{10} I_{\beta} &= \beta \log_{10} \sqrt{2} + \log_{10} \sqrt{2} \\ &= 0.1505 (\beta + 1).\end{aligned}$$

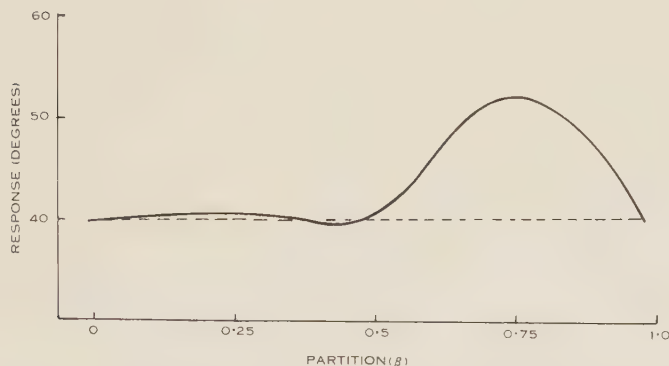


Fig. 2.—Relationship of response to the partition (β) at an arbitrary dose level (η). The broken line indicates the expected response on the basis of simple additivity of the doses reaching the affected cells from both routes of administration.

From the analysis of variance estimates of the mean slopes of the dose response lines corresponding to the five values of β may be obtained. Successive division by the corresponding values of the logarithmic interval reduce these values to common logarithms. When $\beta = 1$, the slope is 45.9, a value typical of the intravaginal route of administration, and when $\beta = 0$ the slope is 91.8, a value typical of the subcutaneous route.

(ii) *Partition* (β).—In the analysis the orthogonal comparisons A and M are not significant. Thus, in this experiment, equivalent doses have been given by both routes of administration, and also the joint dose at $\beta = 0.5$ is also equivalent to these doses. The comparisons N and E are highly significant. Inspection of the data indicates that there is a large synergism between the doses administered jointly at $\beta = 0.75$. While there is a small increase in response at $\beta = 0.25$ it is only of minor importance when compared with the large effect at $\beta = 0.75$, and is not significant if only the first three values of β are included in the analysis.

The data are illustrated schematically in Figure 2, for an arbitrary level of η .

(b) *Experiment 2.* $0.5 \leq \beta \leq 1$ (*Table 3*)

In the previous experiment a large increase in response, and also an increase in the slope of the dose response line, was found in the open interval $0.5 < \beta < 1$. The second experiment was designed to study this region in detail paying particular attention to the slope of dose response lines.

Experiment 1 showed a large increase in mean response in the centre of this region. It was therefore decided to use a special experimental design so that animals in the groups at the centre of this range received smaller doses. The

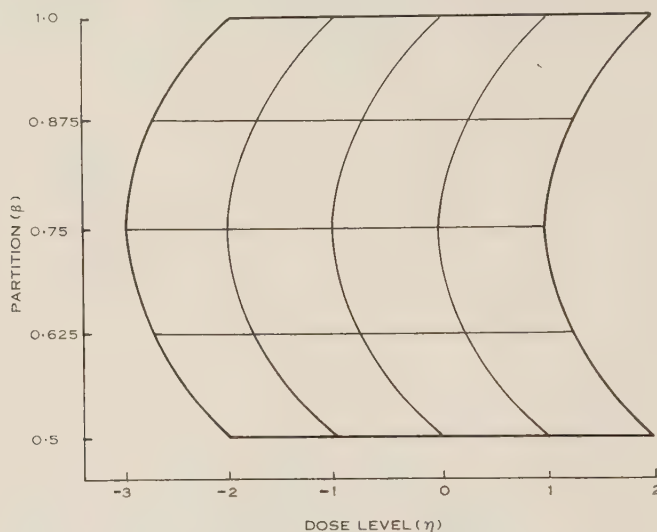


Fig. 3.—The design of Experiment 2.

experimental design is illustrated in Figure 3. It can be seen that the centre groups, corresponding to $\beta = 0.75$, receive log doses reduced by one scale unit of η when compared with the groups at $\beta = 0.5$ and $\beta = 1.0$. The other two values of β in this experiment were 0.625 and 0.875, and received 0.75 of a scale unit less than the extreme groups.

This experimental design is an extension of the parallelogram type of design introduced by Claringbold, Biggers, and Emmens (1953). In this case, doses expected to produce similar responses fall on a parabola. The responses may be related directly to functions of the coordinates of this design. Since, however, these coordinates are not independent the information matrix is not diagonal, even if the angular transformation is used and there are equal numbers of animals per group. Also, the analysis of variance does not consist of independent sums of squares, and hence analysis is very tedious.

The problem is simplified by defining a coordinate function

$$X_1^* = X_1 - 0.25 X_2^2 + 1,$$

which is independent of X_2 . The information matrix corresponding to functions of X_1^* and X_2 is diagonal if (1) orthogonal comparisons or functions, (2) angular transformation, and (3) equal group numbers are used. Since the interval of η

is constant for all dose response lines, this transformation does not affect estimates of slope. The analysis of variance has been made for the data of Table 3 with regard to functions of these coordinates (Table 4).

TABLE 3

PERCENTAGE RESPONSE OF MICE TO THE JOINT ADMINISTRATION OF OESTRONE BY BOTH THE INTRAVAGINAL AND SUBCUTANEOUS ROUTES OF ADMINISTRATION (EXPT. 2, 12 ANIMALS PER GROUP)

Joint Dose of Oestrone		Logarithmic Coding			Response (%)	
Intravaginal (10^{-4} μ g)	Subcutaneous (10^{-4} μ g)	X_1	X_1^*	X_2	Day 1	Day 2
0.59	17.4	-2	-2	-2	0	17
0.06	26.9	-1	-1	-2	33	25
1.90	40.0	0	0	-2	50	75
3.40	59.0	1	1	-2	67	83
6.00	85.0	2	2	-2	100	100
0.39	5.2	-2.75	-2	-1	0	17
0.70	7.1	-1.75	-1	-1	17	33
1.27	9.5	-0.75	0	-1	17	33
2.29	12.7	0.25	1	-1	50	83
4.10	16.9	1.25	2	-1	75	100
0.36	1.6	-3	-2	0	0	8
0.60	2.6	-2	-1	0	8	32
1.10	3.2	-1	0	0	42	50
1.99	4.8	0	1	0	100	100
3.58	6.0	1	2	0	100	100
0.40	0.9	-2.75	-2	1	8	0
0.70	1.1	-1.75	-1	1	8	33
1.28	1.4	-0.75	0	1	67	67
2.31	2.5	0.25	1	1	83	75
4.16	3.2	1.25	2	1	75	92
0.62	0	-2	-2	2	33	17
1.11	0	-1	-1	2	42	25
2.00	0	0	0	2	67	50
3.60	0	1	1	2	75	83
6.48	0	2	2	2	83	83

Constants

$$a_L = 2 \times 10^{-4}$$

$$a_S = 800 \times 10^{-4}$$

$$\eta = X_1$$

$$\beta = 0.5, 0.625, 0.75, 0.875, 1.0$$

$$r_L = 1.8$$

$$r_S = 1.25$$

(i) *Dose* (η).—Only the linear component for doses is significant, indicating that dose response lines are all linear. Investigation of the first order interactions between dose and partition shows that the dose response lines cannot be considered parallel. The first orthogonal comparison for parallelism is between the extreme dose response lines, and is significant. The data show that

TABLE 4

ANALYSIS OF VARIANCE OF THE DATA OF TABLE 3 FOLLOWING THE TRANSFORMATION DEFINED BY

$$X_1^* = X_1 - 0.25 X_2^2 + 1$$

Source of Variation	D.F.	Mean Square	<i>F</i>
Days	1	415	5.5*
Doses (X_1^*)	(4)		
Linear	1	21963	291***
Quadratic	1	17	<1
Cubic	1	193	2.6
Quartic	1	19	<1
Partition (X_2)	(4)		
<i>A</i>	1	0	<1
<i>M</i>	1	0	<1
<i>N</i>	1	482	6.4*
<i>E</i>	1	88	1.2
First order interactions:			
Days \times doses	4	5	<1
Days \times partition	4	145	1.9
Doses \times partition	(16)		
Linear \times <i>A</i>	1	348	4.6*
Linear \times <i>M</i>	1	581	7.7**
Linear \times <i>N</i>	1	28	<1
Linear \times <i>E</i>	1	13	<1
Remainder	12	98	1.3
Other interactions	16	46	<1
Theoretical variance	∞	75.3	

* $0.05 > P > 0.01$.

** $0.01 > P > 0.001$.

*** $P < 0.001$.

the regression on η at $\beta = 0.5$ is greater than the regression at $\beta = 1.0$. The second comparison is highly significant and is due to a large increase in the regression on η at $\beta = 0.75$. The regression coefficients may be converted to common logarithms as before. The relationship of slope to β is illustrated schematically in Figure 4.

(ii) *Partition* (β).—The significance of the *N* effect indicates that while the response in the centre dose response lines was anticipated in setting up the experiment, the response in the second and fourth dose response lines was slightly less than expected. The result confirms unequivocally the synergistic effect observed in the first experiment.

IV. DISCUSSION

In the present problem, angle response is linearly related to log dose of oestrone for both routes of administration. As a working physiological basis we may assume that response is linearly related to the number of molecules of oestrone or weight of oestrone finally reaching the affected cells. It may also be assumed that the difference in slope between the two routes of administration is due to differences in absorption, fate, and excretion rather than different distributions of individual effective number of molecules finally reaching the cells responding. Tissue culture studies of Biggers, Claringbold, and Hardy (1955) have indicated that the effective dose at the cellular level is much smaller than the M.E.D. found using the intravaginal route.

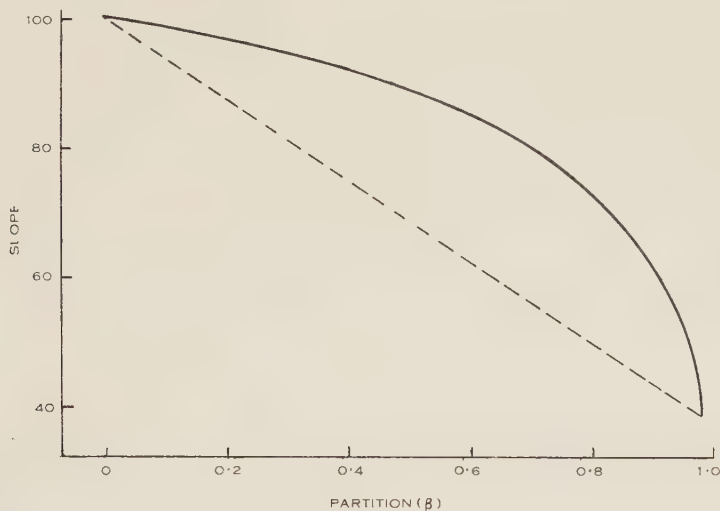


Fig. 4.—Relationship of the slope of the dose response line to the partition (β). The broken line indicates the expected relationship on the basis of simple additivity of the doses reaching the affected cells from both routes of administration.

The linearity of the log dose response line immediately implies that *logarithms of doses* are additive. The simplest hypothesis of simultaneous action of two routes of administration is the additivity of log doses, even though they reach the site of action by different routes. In the above experiments this null hypothesis is disproved by significant departures from linearity of response on β .

The increase in response at $\beta=0.75$ cannot be explained on the basis of additivity of the log dose of oestrone reaching the epithelium by both routes, and it may be said that there is a synergism between the routes of administration used above. Also the experiments indicate that the slope of the dose response line obtained when oestrone is administered simultaneously by both routes is greater than expected on the basis of log additivity of dose. It could be said that there is a synergism with regard to slope for joint administration

(Fig. 3). The point of maximum synergism is $\beta = 0.75$, which corresponds, depending on η , to approximately $\alpha = 0.95$ to 0.98 . Thus the administration of very small doses subcutaneously has a profound effect on the response initiated mainly by intravaginal administration.

The main question to be answered is how can such a small subcutaneous dose produce its effect? It is possible that the subcutaneously administered oestrone stimulates blood flow to the vagina and also produces changes in the vaginal connective tissue. While the intravaginal portion of the dose is primarily concerned with initiation of the response, the subcutaneous portion of the dose may facilitate the passage of nutrients to the vaginal epithelium. Studies of the mitotic rate of the epithelium following oestrogen administration have shown a great increase in cellular activity during the 48 hr after injection of oestrogen (Biggers and Claringbold 1955*b*). The work of Claringbold (1953) with inhibitors shows that active metabolic processes are associated with the vaginal response. It is reasonable therefore to assume that the final morphological response may be influenced by the supply of nutrients from the circulation. In view of the small subcutaneous portion of the dose at maximum synergism it seems unlikely that the effect is due to the stimulation or depression of another endocrine system.

Further, it seems possible that the synergistic effect on slope is another manifestation of the effect suggested above. More work on this problem is required, especially with regard to the relationship between the epithelium and its underlying connective tissue.

V. ACKNOWLEDGMENTS

The authors wish to acknowledge the advice of Professor C. W. Emmens during the course of this work. The expenses of the work were defrayed by grants from the Wool Industry Fund and the Commonwealth Bank of Australia.

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GROWTH OF THE MOUSE COAT

IV. COMPARISON OF NAKED AND NORMAL MICE

By A. S. FRASER* and T. NAY*

[*Manuscript received January 19, 1955*]

Summary

The movement of waves of hair growth is very regular both in pattern and timing in Naked mice. Although normal mice do not differ markedly from Naked mice in the pattern and timing of the first and second cycles of hair growth, the pattern and timing of subsequent growth waves in normal mice is extremely irregular.

I. INTRODUCTION

Our studies of waves of hair growth in Naked mice (Fraser and Nay 1953; Nay and Fraser 1954) have shown (*a*) that the waves move in a very regular pattern from head to tail, (*b*) that the interval between successive cycles is constant for each region, and (*c*) that this interval increases in a gradient from head to tail. This regularity is independent of age and season, indicating that the cycling of hair growth is independent of environmental control. An alternative explanation of the regularity is that stress caused by the nakedness has increased environmental sensitivity to an extent which causes the initiation of growth waves at the maximum rate. This can be checked by an equivalent study of hair growth cycles in normal mice.

Previous studies on cycles of hair growth in normal mice are not sufficiently extensive to allow comparison with our data on Naked mice. Dry (1926) dissected a series of dated skins, and determined the progression of waves of hair growth. He found that the third cycle differed from the first and second and that in later cycles the progression of growth waves was irregular. Although his data are not given quantitatively, he states that the interval between successive cycles was much greater and highly variable. Since this is markedly different from the pattern of hair growth in Naked mice, a study was initiated of hair cycles in normal mice. Soon after completion of this study, Borum (1954) published a comprehensive account of hair cycles in normal mice which extends Dry's (1926) observations and agrees very closely with our own data. Differences and similarities will be discussed below.

II. MATERIALS AND METHODS

Three stocks of normal mice were used: (*a*) the NA stock described previously (Fraser and Nay 1953), (*b*) the F_1 of the cross of the NA stock with

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the C₅₇ inbred line, and (c) the OS stock of normal mice from a selection experiment for oestrogen sensitivity. The bulk of the data was collected from NA mice. The NA \times C₅₇ mice were used to compare the initiation of hair growth in Naked and normal mice. The OS mice were used in a comparison designed to determine whether clipping had any effect on hair growth waves.

Naked mice were described as detailed previously (Fraser and Nay 1953). Normal mice were clipped with electrical clippers and the appearance of hair growth scored on the same scale as in Naked mice. The data are represented as position on a scale of 64 units, measured along a line from the head (0) to the root of the tail (64), along the side of the animal.

Since any differences between Naked and normal mice could be due to effects of clipping, 130 adult male mice of the OS stock were divided into two groups, within litters, to test the effect of clipping. One group was clipped with electrical clippers over the main body area every time hair growth was

TABLE I

DIFFERENCES BETWEEN THE NUMBERS OF NAKED AND NORMAL MICE SHOWING ERUPTION OF GUARD AND COAT HAIRS AT DIFFERENT AGES

Type of Hair Growth	Type of Mice	Age in Days from Birth											
		1	2	3	4	5	6	7	8	9	10	11	12
Guard	Normal	—	—	5	7	3	—	—	—	—	—	—	—
	Naked	—	—	—	9	5	2	—	—	—	—	—	—
Coat	Normal	—	—	—	—	—	4	8	3	—	—	—	—
	Naked	—	—	—	—	—	—	1	4	8	—	1	2

noted during 3 weeks. Then all the mice were killed, skinned, and the skins stained and cleaned, allowing the positions of the growth waves to be determined, usually with precision, and only rarely with difficulty. Both in the clipped and non-clipped groups, the patterns of position of the growth phase bore no resemblance to these characteristics in Naked mice. No qualitative effect of clipping could be detected, but the possibility of a quantitative effect cannot be excluded. The extremely regular pattern of hair cycles in Naked mice contrasts so greatly with the extremely irregular patterns in normal mice, that any quantitative effect of clipping will not effect the conclusions on the effect of the Naked gene.

III. RESULTS

(a) *Initiation of the First Cycle*

The emergence of the tips of hairs above the skin can be detected 1 or 2 days after birth, but the main bulk of the coat does not emerge until 8 days after birth (Dry 1926; Fraser 1951; Grüneberg 1952). No similar observations

had been made on Naked mice and therefore a series of litters from the cross of N/+ males of the NA stock with +/+ females of the C₅₇ inbred line were scored for emergence of hair during the first cycle of growth.

The mice were observed with a dissection microscope. Groups of 15 normal and 16 Naked mice were scored for eruption of guard and coat hairs. The results are given in Table 1, which shows that Naked mice lag behind normal mice in the eruption of both guard and coat hairs by an average of 1-2 days. A feature of Naked mice was the frequent low density of guard and coat hairs.

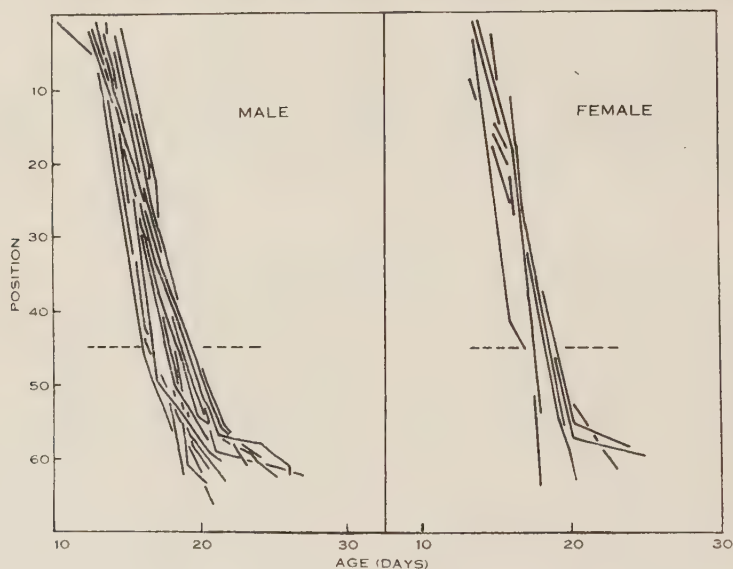


Fig. 1.—Cessation of growth of first hair cycle in Naked mice.
 ----- Cessation of growth of first hair cycle in normal mice.

(b) Cessation of Growth of the First Cycle

The clipping method does not allow determination of the time of cessation of hair growth, whereas this can be easily scored in N/+ mice. The graphs of age of cessation of growth during the first cycle, plotted against position, are shown in Figure 1. Fraser (1951) found from measurement of the lengths of hairs that the growth of the hairs of the first cycle ceased at 18½ days in the A and CBA inbred lines of mice. Since his results only apply to the mid-back region, the equivalent region on our scale is indicated in Figure 1. In these N/+ mice the age at cessation of hair growth of the first cycle at this region was 18·8 days. The difference is small considering that the two estimates were determined by quite different methods on mice grown in different animal houses whose management systems were not identical.

(c) Initiation of Growth of the Second Cycle

The results obtained by Nay and Fraser (1954) show that the progression of a growth wave from head to tail in Naked mice is regular, and they found

few exceptions to this. To compare Naked and normal mice, a series of litters from the NA stock segregating for the Naked gene, was studied. The sequence of initiation of growth of the second cycle is shown in Figure 2, separated into litters. The frequency of mice showing deviations from the steady progression is greater than in the previous study, but there is no indication that differences occur between Naked and normal mice in the frequency of aberrant patterns of growth of the second cycle. Further, there are no qualitative differ-

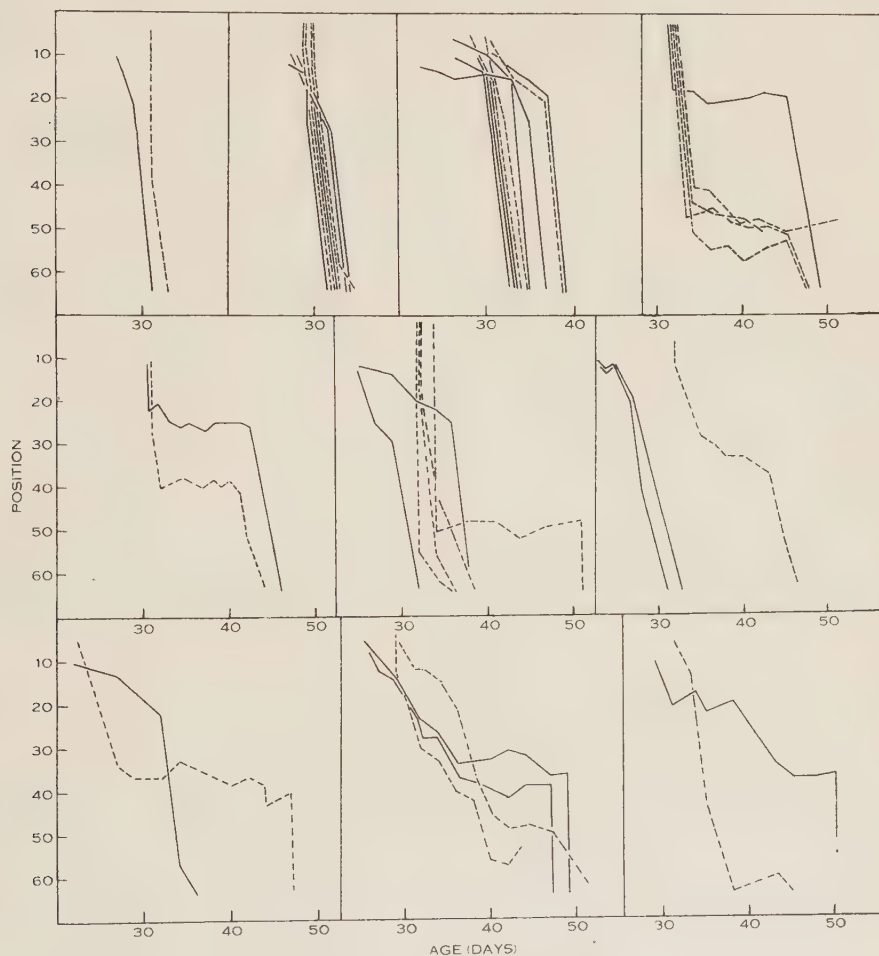


Fig. 2.—Initiation of growth of second hair cycle in several litters of Naked and normal mice. — Normal mice. ---- Naked mice.

ences in timing or pattern between Naked and normal mice. Finally, there is a strong indication that the occurrence of an aberrant pattern is general over the mice of a litter, both Naked and normal.

If these data are averaged for all mice, a quantitative difference between Naked and normal mice can be detected. In Table 2 the age at initiation of

the second cycle is given for several positions along the body averaged (i) for all mice, and (ii) for mice with regular patterns. There is a general agreement between the respective averages for Naked and normal mice. Excluding mice with irregular patterns, it is clear that the age at initiation of the second

TABLE 2
AVERAGE AGE AT INITIATION OF THE SECOND CYCLE AND INTERVAL BETWEEN FIRST AND SECOND CYCLE

Position	Average Age at Initiation of Second Cycle (days)				Interval between First and Second Cycles (days)	
	All Mice		Mice with Regular Patterns			
	N/+	+ / +	N/+	+ / +	N/+	+ / +
20	31.95	30.95	31.42	28.54	21.42	20.54
30	32.86	33.66	32.26	29.63	22.26	21.63
40	34.63	36.04	32.77	30.27	22.77	22.27
50	38.68	36.61	33.30	31.18	23.30	23.18
55	41.04	36.90	33.36	31.36	23.36	23.36

cycle is about 2 days more in Naked than in normal mice. This lag in Naked mice parallels the lag in the initiation of the first cycle, and if we compare the interval between the first and second cycles (shown in Table 2), it is clear that no real difference occurs between Naked and normal mice for this statistic.

TABLE 3
INTERVAL BETWEEN SECOND AND THIRD CYCLES IN NORMAL MICE: AVERAGES OF 12 MALES AND 9 FEMALES

Males		Females	
Position	Days	Position	Days
20	40.94	20	43.8
30	45.97	30	46.0
40	50.02	40	47.9
50	49.22	50	47.8
55	47.27	55	45.3

(d) *Initiation of the Third and Subsequent Cycles*

The initiation of successive hair cycles can be most easily seen in graphs like that shown in Figure 3, in which the sequence of initiation is plotted against position separately for all Naked mice. The "irregular" mice are included, and

even though they confuse the pattern, they do not obscure the occurrence of cycles of hair growth at regular intervals. The same type of diagram is shown in Figure 4 for normal mice.

(e) *Interval between Successive Cycles in Normal Mice*

The interval between the second and third cycles is given for several positions in normal mice in Table 3. No real difference due to sex can be detected. Amongst 21 animals there was no significant correlation at position 40 between the intervals between the second and third and the third and fourth hair cycles.

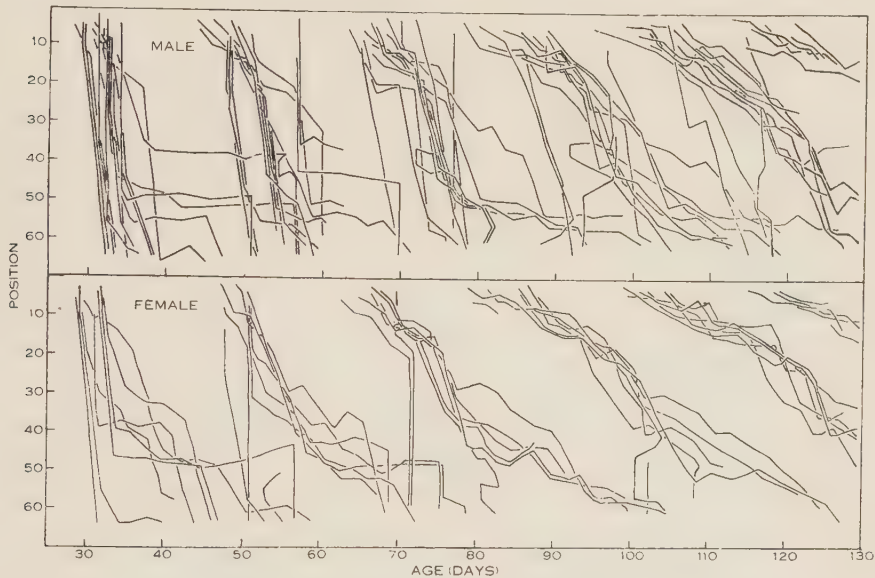


Fig. 3.—Hair growth cycles in Naked mice.

The third cycle of hair growth in normal mice is highly variable both in its timing and in its pattern of movement over the body. This variability is qualitatively greater than that found in Naked mice. Due to the long interval between successive cycles in normal mice, not many mice were scored for the fourth cycle. The pattern and timing of this cycle was as variable as the third cycle. The data are not shown in Figure 4, since they would only further complicate an already complicated diagram.

IV. DISCUSSION

The Naked gene, although scored by its effect on the retention of the coat, causes a number of other effects, summarized by Grüneberg (1952). In this paper two further effects of the gene have been identified. These are (a) a lag of 1-2 days in the initiation of the first coat, and (b) a maintenance of

regular cycles of hair growth, whereas in normal mice the pattern and timing of hair growth is irregular after the second cycle. The latter effect could be due to the nakedness caused by the shedding of completed hairs. The large areas of uncovered skin almost certainly place a stress on the metabolic system, and, if the occurrence of hair cycles is regulated by the occurrence of such stress, then Naked mice should maintain the initiation of hair cycles at a maximum rate. This can be tested by experiments involving the application of temperature stress to normal mice.

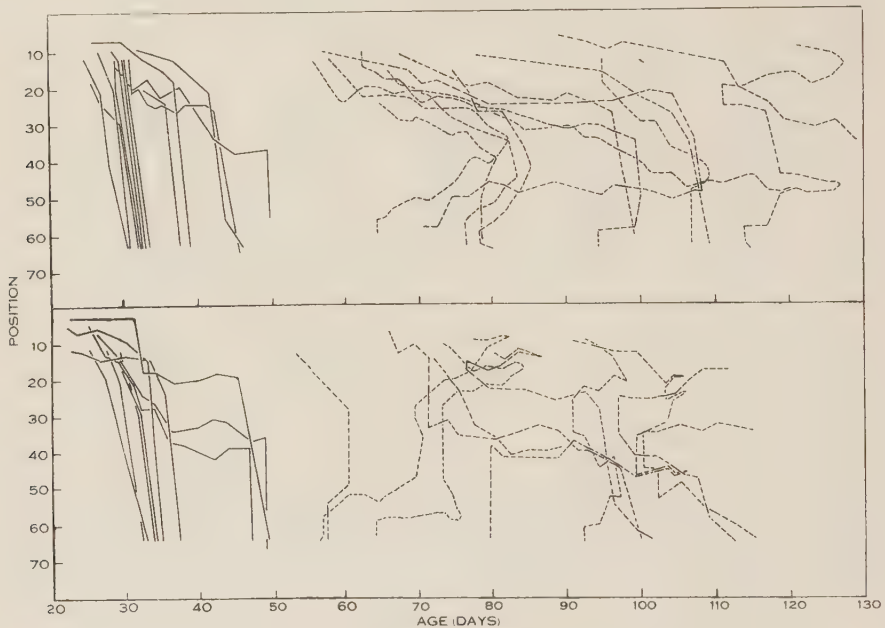


Fig. 4.—Hair growth cycles in normal mice. ——— Initiation of second cycle. - - - - Initiation of third cycle.

Borum (1954) dyed albino mice and then scored the occurrence of hair growth waves from the appearance of white hairs. Her estimates of the ages of initiation of the first and second cycles agree with ours, and she also noted the occurrence of irregular patterns of movement of the second growth wave as in our material. She states "that in a few instances the growth wave of G_2 may suddenly cease for varying periods of time; this may occur at any stage of its development. This phenomenon is reflected by an unmistakable difference of colour in the coat, the growth of G_2 in one area being complete before it starts in the adjacent area."

In our data there is a suggestion that the timing and patterns of the third cycle differ between sexes in normal mice. Borum did not note any difference although she states that the duration of spread of the individual hair generations increased with age and was longer in females than in males. This had been demonstrated by Emmens (1942).

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GROWTH OF THE MOUSE COAT

V. EFFECTS OF PREGNANCY AND LACTATION

By T. NAY* and A. S. FRASER*

[*Manuscript received February 9, 1955*]

Summary

Waves of hair growth move regularly from head to tail in Naked mice. This movement ceases at late pregnancy, and does not resume until about 12 days after birth in non-suckling dams, and 35 days in suckling dams.

I. INTRODUCTION

The growth of the mouse coat is a cyclic process characterized by waves of hair growth, which in Naked mice move regularly from head to tail. The location of waves of hair growth can be found only with difficulty in normal mice. However, in Naked mice, a hair breaks off at skin level after completion of its growth, and therefore the bands of hair on Naked mice represent the location of waves of hair growth. Some aspect of pregnancy disrupts the hair growth cycle (Lebedevsky and Dauvert 1927; Dannel and Kahls 1947; Fraser and Nay 1953). Previous descriptions were based on only a few animals and did not use a quantitative method. It is not difficult to describe the hair growth cycles quantitatively (Fraser and Nay 1953), and in the present study a more comprehensive description of the effects of pregnancy and lactation is given in quantitative terms.

II. MATERIAL AND METHODS

The mice were all of the NA stock. This stock and the methods of scoring the positions of the hair bands have been described previously (Fraser and Nay 1953). The hair bands are located on a line running along the side of the animal and which is divided into 64 units, and position is measured from the head (0) to the root of the tail (64). Results are expressed as graphs of position against age, showing the progression of waves of hair growth.

Virgin N/+ females were mated to normal males, which were removed before parturition in most of the matings. In some females the litter died or was killed within 2-3 days of birth. In others the litter was weaned normally at 20 days. Males were left in a few of the matings to allow successive litters.

III. RESULTS

(a) *General Description*

The effects of pregnancy and lactation are shown in Figure 1, which gives the hair cycle graphs of four mice, one from each group of the study. The

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progression of hair bands ceases approximately at the birth of the litter. Some aspect of pregnancy, therefore, inhibits the initiation of the growth phase in regions which are potentially ready to enter the growth phase. In some mice, a retention of hairs beyond the time at which they are usually shed occurs in those regions which were actively growing hair at the time of inhibition. If the litter is killed soon after its birth, hair growth in the dam is initiated simultaneously all over the body about 12 days after the birth of her litter, except in a region corresponding to the position of the hair band at the time of inhibi-

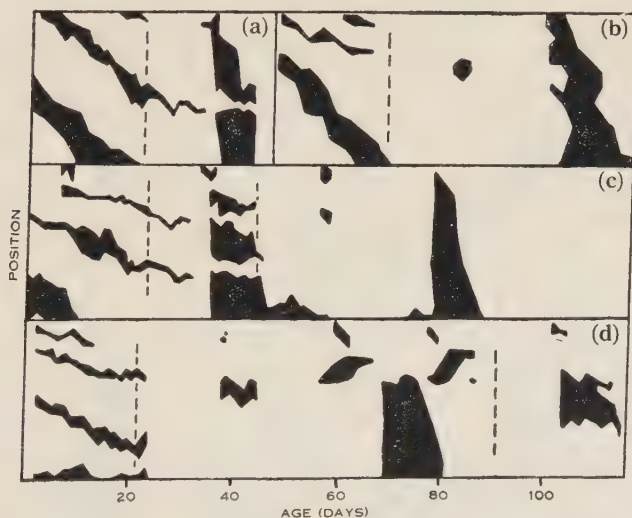


Fig. 1.—Position of hair bands plotted against age in days. Dotted lines show time of birth of litter. (a) Litter killed soon after birth, no subsequent pregnancy allowed. (b) First litter suckled and weaned, no subsequent pregnancy allowed. (c) First litter killed, the second litter suckled and weaned. (d) First and second litter suckled and weaned.

tion. If the litter is suckled and weaned normally hair growth in the dam is initiated all over the body about 35 days after birth. Prior to this, hairs are formed in a region which corresponds in its position to the location of the hair band at the time of inhibition. These features are shown in a simple form in Figure 2. In subsequent sections, each of these aspects will be considered in detail.

(b) *Inhibition of the Initiation of the Growth Phase*

The age at which the movement of a hair band stops can be estimated from the intersection of a regression line fitted to the rate of movement of the hair band, and a line fitted to the average position of the retained hair (Fig. 3).

The average time at which inhibition occurs is 2.9 days after the birth of a litter, but the range is fairly wide, ranging from 7 days before to 4 days

after birth. There is a tendency for bands located near the head, and on the fore-shoulder, to show inhibition earlier than those located in the main body region. In some mice several bands occurred and were affected by the pregnancy inhibition. There is no indication of any correlation between the different bands in the age at which their movement is inhibited.

(c) Retention of Hairs

Apart from pregnant mice, no Naked mouse has been observed to retain coat hairs beyond completion of their growth. Retention, although not a universal feature of the effects of pregnancy, occurred in 22 out of 25 mice, and 38 out of 60 hair bands. Excluding those hair bands which showed no retention,

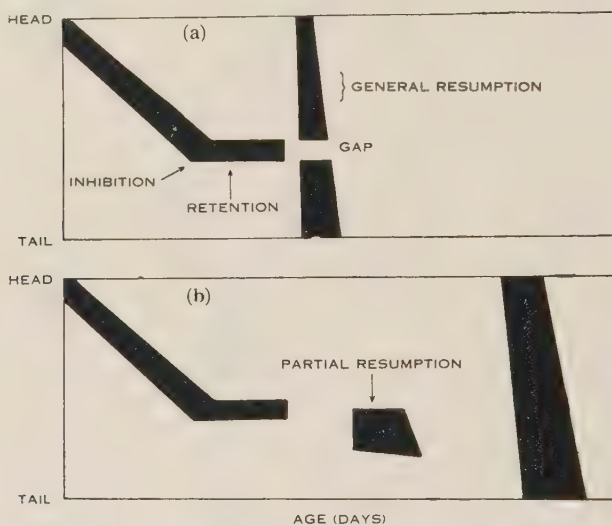


Fig. 2.—Simplification of the effects of pregnancy and lactation on hair growth. (a) General pattern in the absence of lactation. (b) Added effects of lactation.

the average duration of retention was 6.8 days. An interesting feature of retention is that in some of the mice the termination of retention coincides with the initiation of general hair growth. We use the term "retention" meaning that the hairs are retained statically after the time at which they would normally have been shed. Another possibility is that the follicles in those regions could be maintaining the growth phase. Since the hairs of "retained" regions do not appear to be any longer than normal, this latter possibility appears unlikely.

(d) General Resumption of Hair Growth

The cessation of inhibition in non-suckling females is usually marked by a general initiation of hair growth, except in those regions corresponding to the location of the hair bands at the time of inhibition. General resumption occurs at an average of 12.1 days after birth, ranging from 8 to 14 days.

If the female is suckling a litter, a small region of the skin resumes hair growth at about 9.3 days after birth, followed by a general resumption of hair growth all over the body at an average of 35.3 days after birth, ranging from 31.0 to 42.0 days.

A second pregnancy beginning at the first post-partum oestrus does not interfere with general regrowth of hair provided lactation does not occur (Fig. 1 C). Further, lactation does not interfere with the general resumption of hair growth if the number of young is small (1-3).

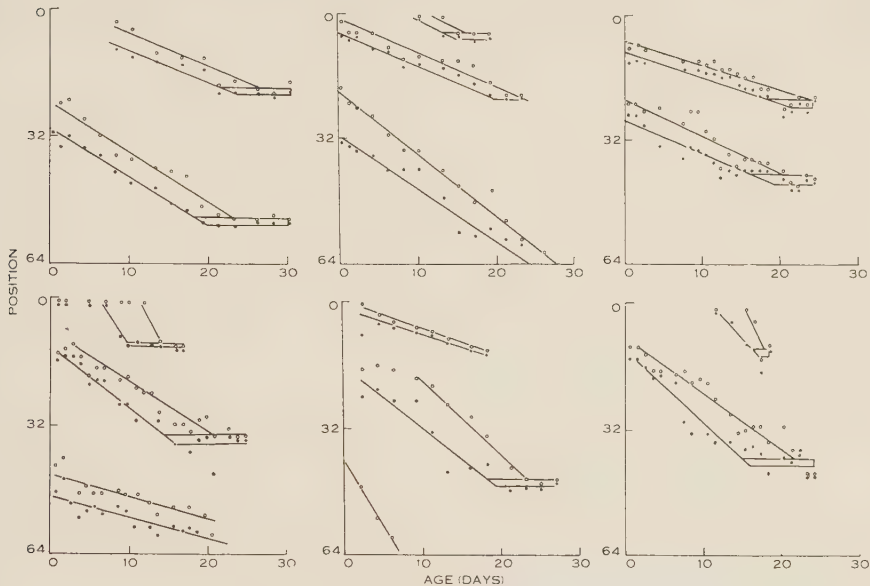


Fig. 3.—Positions of hair bands plotted against age in days. O Regressions of the rate of movement of the bands (visual fitting). ● Regressions of the position of retained hairs (visual fitting).

(e) Gap in the General Resumption of Hair Growth

In those mice whose litter died or was killed at birth, hair growth occurs all over the body except in limited regions, which are termed "gaps." The position of these gaps corresponds closely with the position of the hair bands at the time of inhibition. This is illustrated in Figure 4, which gives a correlation diagram of the position of hair band plotted against the position of the gap.

Similarly in mice suckling a litter, a partial resumption of hair growth occurs in a position related to the position of the hair band at the time of inhibition. Unlike the gap, this region of partial resumption does not correspond closely in position to that of the inhibited hair band. In general it is located nearer the tail, and is wider.

IV. DISCUSSION

Broadly, the above results show that the inhibition of hair growth occurs a few days before birth, and that the resumption of hair growth is affected by the occurrence of lactation, since it does not occur until 35 days after birth of the litter in suckling dams, whereas in non-suckling dams it occurs much earlier, 10 days after birth of the litter.

These facts can be explained if the inhibition is caused by one of the lactation hormones. In a non-suckling dam, such a hormone will cease to be formed soon after loss of the litter, and the lag between disappearance of the hormone and initiation of hair growth can be taken to be 10 days. The interval of 35 days between inhibition and general resumption can be therefore divided

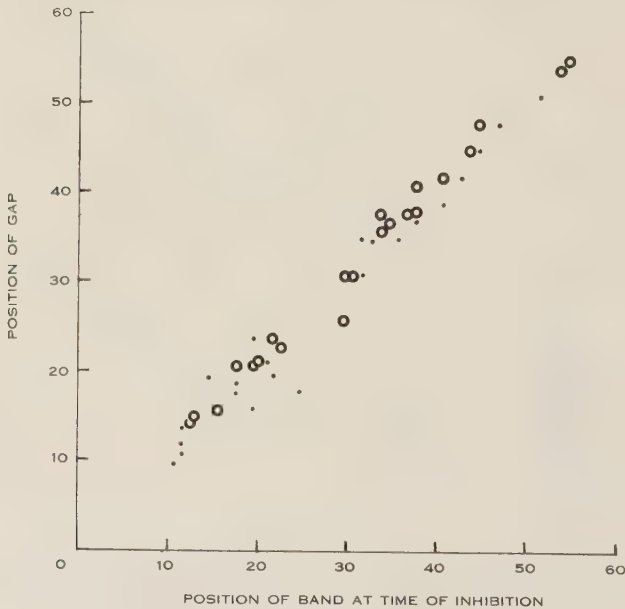


Fig. 4.—Correlation diagram of positions of cranial (dots) and caudal (circles) edges of the gap and of the band at the time of inhibition.

into a period of 25 days of active inhibition and a 10-day lag after the removal of the inhibition following the weaning of the mice. Since mice are certainly weaned before 25 days, a period of 5-10 days probably occurs during which the lactation hormone is gradually disappearing. Some evidence in favour of this can be found in the lapse of time, about 20-25 days, after parturition before normal oestrous cycles are initiated.

The identification of the specific hormone concerned has been attempted by Dannel and Kahls (1947) who found that proluton caused the inhibition of hair growth cycles. However, since these results are based on few animals, the need for a more extensive investigation of the effects of hormones on hair cycles is indicated.

In addition to the correlation of hair growth cycles and hormonal systems, our data indicate that the cycle of hair growth can be divided into different periods which differ in their response to the inhibitory hormone. Comparing the width of a hair band, and of the retained hairs, it is obvious that retention only occurred in the caudal follicles which have just begun growth of their hairs. The data above indicate that follicles which are well into the growth phase are unaffected by the inhibitory hormone, and continue the growth and shedding of their hairs without any change of timing, whereas follicles which have only just begun growth of their hairs are affected, as shown by their retention of the hairs beyond their usual time of shedding.

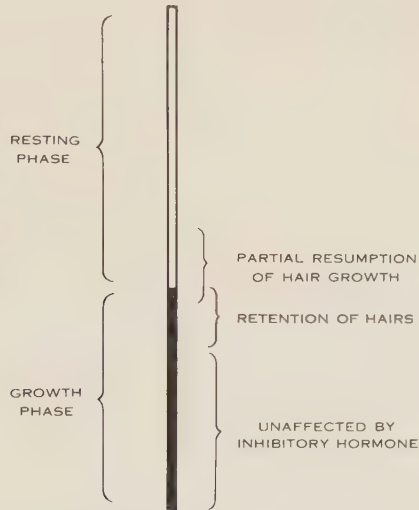


Fig. 5.—Division of a hair growth cycle into periods, as shown by differences in the position of the retained hairs, and in the position of partial resumption of hair growth.

A further sub-division is evident from the positions of the regions of partial resumption which occur at about 12 days after birth in suckling dams. These regions are under the hair band at the time of inhibition, and occur slightly nearer the tail. This shows a difference between follicles in their response to this particular effect of the inhibitory hormone. Partial resumption appears to occur only in follicles which have just begun the formation of their hairs, and in follicles, which, although not in the growth phase at the time of inhibition, were about to produce visible hairs. The sub-division of a hair growth cycle is illustrated in Figure 5.

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A COMPARATIVE STUDY OF GROWTH AND NUTRITION IN BARLEY AND RYE AS AFFECTED BY LOW-WATER TREATMENT

By R. F. WILLIAMS* and R. E. SHAPTER†

[Manuscript received March 31, 1955]

Summary

A comparative study of growth and nutrition in barley and rye was made with two water treatments. The low-water treatment was of an intermittent character and harvests were made after each of the five periods of water stress. Yield reductions due to low-water treatment were highly significant at all five harvests and for both species. The severity of the effects on various plant parts was conditioned by the stage of development of those parts.

The importance of leafiness, as measured by the leaf weight ratio, is stressed as a factor determining interspecific and treatment effects on transpiration rate and the transpiration ratio. An increase in the ratio of roots to shoots has been regarded as a typical response of plants to low-water treatment, but the *immediate* response in the present experiment was a reduction in the root weight ratio. This effect was later reversed in barley but not in rye. The inflorescence weight ratio was reduced by treatment in barley but not in rye. Stem height was unaffected by low-water treatment in rye but was reduced in barley.

The data were examined by classical growth analysis procedures and it was shown that, for successive harvest intervals, net assimilation rates on a leaf nitrogen basis were depressed by treatment in accordance with its severity. There was a tendency for nitrogen to be excluded from the leaves and to accumulate in the "stems" as a result of wilting. Phosphorus intake by the shoots was considerably reduced by low-water treatment, and there were initial decreases in relative leaf phosphorus for both species. This effect was reversed later. Relative contents of silica also tended to be reduced by treatment. The relative contents of potassium, calcium, and magnesium were increased by treatment in the tissues of barley, but these effects were less pronounced in rye. Rye absorbed very much less sodium than did barley, and there were chemically equivalent but relatively smaller differences in the intake of chlorine. There was also evidence for selective absorption of manganese, relative contents being higher in rye than in barley.

The results and the relevant literature are discussed in terms of the growth patterns of the experimental plants. It was found that those plant parts which are growing most actively during the period of low-water treatment are those which suffer the greatest check to their growth and it is indicated that many of the effects on the ratios of plant parts are no more than indirect consequences of this fact. In other cases, such effects could arise directly from the action of water shortage on morphogenetic processes.

Distribution indices for dry weight change are examined, and for many cases there were remarkably small immediate effects of low-water treatment. The exceptional behaviour of rye in this respect is thought to be relevant to its drought resistance.

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I. INTRODUCTION

It is now well recognized that a number of factors contribute to drought resistance in plants. Some of these, such as depth of root system, can be studied properly only in the field. We know much less, however, about the physiological characters which enable the plant to withstand severe desiccation, and these factors can rarely be studied effectively in the field. The elucidation of these physiological characters calls for detailed comparative studies of plants possessing well-established differences with respect to drought resistance.

Cereal rye undoubtedly possesses drought-resistant qualities in a high degree, and this paper is based on an experiment in which it was compared with barley, which is not usually regarded as resistant. The work arose indirectly from an experiment designed to investigate the ability of various plant species to grow on and stabilize drifting sand at Pallamana, South Australia. Barley and rye were the only plants to establish themselves successfully, and only the rye survived the combined effects of drought and sand drift (Waite Institute Report 1938, p. 28). Since that time rye has been used quite extensively in southern Australia for sand-drift control (Herriot 1947-8; Sims 1949).

It may well be that the characters which enabled rye to survive under these extreme conditions are also such as cannot adequately be studied in pot culture, but it was hoped that a comparative study of the two species would reveal differences of response to low- and high-water treatment. This did, in fact, prove to be the case, and to that extent the experiment can be regarded as a contribution to the complex problem of drought resistance.

Perhaps the main value of the data to be presented lies in the fact that, for a considerable portion of the life of two plant species, we have accurate information concerning both relative and absolute contents of no less than nine chemical elements, most of which are essential nutrients and all of which are normally present in plants. It is true that the low-water treatment effects on these plant constituents are difficult to interpret with any confidence, but they do shed some light on certain anomalies which exist in the literature concerning the effects of such treatment on nutrient accumulation in plants (Wadleigh and Richards 1951).

II. EXPERIMENTAL METHODS

(a) General

One hundred enamelled pots containing approximately 14 kg of a 2:1 mixture of Waite loam and washed river sand were used for the experiment. Half of these were seeded with barley, *Hordeum vulgare* L. and half with rye, *Secale cereale* L. on April 17, 1939, and the pots mulched with fine gravel to reduce water loss from the surface of the soil. At an early stage the plants were thinned to five uniform seedlings per pot. Until June 5 (day 49) all pots were watered frequently and so as to bring them back to 65 per cent. of the water-holding capacity of the soil. This procedure is not entirely satisfactory, but it was standard practice at the time of the experiment. Field capacity for the soil mixture would have been somewhere between 50 and 60 per cent. of the

water-holding capacity, but soil water would not long have remained above the field capacity after each watering.

After June 5, half of the pots were subjected to a low-water treatment, and were watered to the original level only on July 3 and 24, August 14, and September 4 (days 77, 98, 119, and 140). This gave four treatments, and on five occasions, July 3 and 26, August 16, and September 6 and 27 (days 77, 100, 121, 142, and 163), five pots of each treatment were harvested. For harvests 2, 3, and 4 it will be noted that there is a discrepancy of 2 days between date of watering-up and date of harvest. Only pots for later harvests were watered on the first of these pairs of dates. Low-water pots to be harvested 2 days later were not watered. This unfortunate expedient tends to destroy the strict continuity of the data for the low-water treatments, but not for the high-water treatments.

At each harvest the leaves were separated at the ligule and, from harvest 3 onwards, the inflorescences were separated from the stems. The stem fraction was a composite one and included leaf sheaths as well as the true stems. Roots were carefully washed from the soil and their dry weights were corrected for soil contamination. Both fresh and dry weights were obtained for the above-ground parts. For the chemical analyses, methods for which are briefly stated below, the inflorescences were reunited with the stems. For the sake of brevity this reunited material will be referred to as "stems".

Pot weight records were kept throughout the experiment and transpiration losses were calculated after correcting for the fresh weights of the plants and for losses from the soil surface.

(b) Chemical

(i) *Ash*.—While data for total and soluble ash are of relatively little value by comparison with data for individual elements of which they are composed, they do give a measure of the total inorganic material in these categories. The total ash values of Figure 5 are carbonate-free, and the usual precautions were observed in the preparation of the ash. Soluble ash is that portion of the total ash remaining after subtraction of silica plus free carbon and carbon dioxide (if present). Insoluble ash gives a sufficiently accurate estimate of plant silica.

(ii) *Calcium*.—As a preliminary to this estimation, phosphoric acid, iron, and aluminium were removed by precipitation with ammonia after addition of sufficient ferric chloride to ensure complete removal of phosphate. Manganese was removed by the use of bromine and ammonia. The calcium was precipitated as oxalate and subsequently titrated as usual after solution of the oxalate in dilute sulphuric acid.

(iii) *Magnesium*.—Magnesium was precipitated as magnesium ammonium phosphate in the filtrate from the calcium determination by the usual procedure and with the usual precautions. The magnesium ammonium phosphate was ultimately ignited to magnesium pyrophosphate.

(iv) *Potassium*.—Potassium and sodium were separated as mixed chlorides, the potassium being converted to perchlorate and weighed as such, or in some

cases estimations of potassium were made by precipitating as cobaltinitrite and subsequently converting to perchlorate.

(v) *Sodium*.—Sodium was obtained by difference from the mixed chloride result, or directly by precipitation with uranyl magnesium acetate.

(vi) *Chlorine*.—Chlorine was estimated by the electrometric method devised by Best (1929) and the results checked against the more usual Volhard method after ashing with lime. The values are high for plant material other than chenopodiaceous shrubs, but there seems no reason to doubt their validity, especially as the ash contents are also high. It has been suggested that watering the plants with tap water, which was relatively high in salts, may have been partly responsible.

(vii) *Manganese*.—Manganese was estimated by the colorimetric periodate method.

(viii) *Nitrogen*.—Nitrogen was estimated by the Kjeldahl method, using selenium as the catalyst.

(ix) *Phosphorus*.—Phosphorus was estimated by the gravimetric Lorenz method.

III. PRESENTATION OF DATA

(a) *The Severity of Treatment*

As might have been expected, the interpretation of the results of this experiment is complicated by the necessity to make interspecific comparisons within a soil-water treatment which is unavoidably lacking in constancy. This is so because of the impracticability of maintaining soil water at levels below field capacity. The expedient of submitting plants to intermittent periods of water shortage, as in the present experiment, has the virtue that it imitates more or less accurately what does in fact happen in the field, but the growth characteristics of the plant species to be compared will almost certainly modify the severity of one or more of the periods of water shortage. Species is, to that extent, confounded with water treatment, and the comparison of quantitative attributes of growth and nutrition may be quite spurious. While such a criticism is very obvious with soil-water treatments it is nevertheless applicable to most interspecific comparisons, especially when pot-culture techniques are used.

Some idea of the effect of species on soil-water treatment in the present experiment will be gained from Table 1, which shows for each harvest interval the number of days during which soil water had been reduced below certain arbitrary levels. This information was derived from pot weights and by using a correction for the fresh weights of the plants. At 600 ml per pot the soil was at the permanent wilting percentage, and it will be noted that the plants succeeded in reducing this to less than half during the later harvest intervals. The picture for the case of barley only is presented diagrammatically as part of Figures 1, 2, and 3. Table 1 shows that, for the period prior to harvest 1, the barley plants were subjected to a much more severe water shortage than

were the rye plants. The differences for intervals 1-2 and 2-3 were in the same direction although less marked, but the difference was reversed slightly for

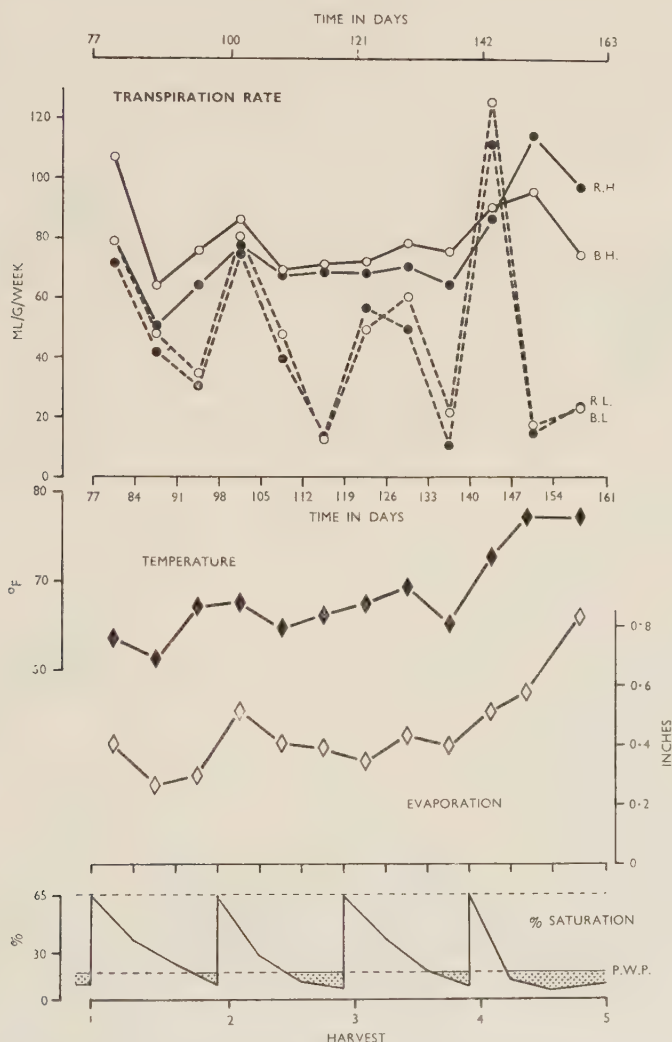


Fig. 1.—Transpiration in ml per g dry weight of leaves per week. The treatment legend is given in Figure 2, but on all figures B.H. = barley, high water, B.L. = barley, low water, R.H. = rye, high water, and R.L. = rye, low water. The temperatures are mean daily maxima within the glasshouse, and the evaporation values are expressed in inches of water per week from an evaporimeter at the open end of the glasshouse. At the bottom of the figure and also in Figures 2 and 3 is a diagram representing the soil-water sequence for the barley low-water treatment. The upper limit of watering, 65 per cent. saturation, was somewhat in excess of the field capacity.

interval 3-4. There was little or no difference in water treatment for interval 4-5. Table 1 also shows that for barley the second and fourth intervals were

considerably less severe than the other three. For rye, only the second interval was markedly less severe than the others.

It must be admitted that, in general, the low-water treatment turned out to be more severe for barley than for rye. This difference might have been reduced if, instead of having five plants per pot in each case, there were more rye than barley plants per pot. Caution is clearly necessary in comparing the responses of the two species to low-water treatment, and for this reason attention will be concentrated on effects which seem to have general validity.

(b) Transpiration Rate

Transpiration rates are presented as water loss per unit dry weight of leaf in Figure 1. To obtain weekly values it was necessary to use interpolations on the leaf weight curves.

TABLE 1
TIME IN DAYS THAT SOIL WATER WAS REDUCED BELOW SPECIFIED LEVELS

Soil Water per Pot	Harvest Interval				
	0-1	1-2	2-3	3-4	4-5
Barley, low water					
At or below 600 ml	15	5	11	7	18
At or below 500 ml	13	3	10	6	17
At or below 400 ml	10	1	8	4	16
At or below 300 ml	—	—	5	2	14
Rye, low water					
At or below 600 ml	11	4	10	9	18
At or below 500 ml	9	2	8	8	17
At or below 400 ml	—	—	5	6	15
At or below 300 ml	—	—	—	2	11

For the control plants, except at late maturity, the transpiration rate is lower for rye than for barley. Williams (1935) found for oats that transpiration rates on this basis were significantly correlated with the nitrogen contents of the leaves. The nitrogen content data of Figure 4 show, however, that such a relation is not present here. Indeed, leaf nitrogen is consistently higher in rye than it is in barley.

As is to be expected, rates within the low-water treatment are governed very largely by soil-water level, though immediate past history seems also to have an effect. The first, fourth, seventh, and tenth weeks of the transpiration record immediately follow the harvesting occasions, and for these weeks the soil-water supply was the same in both treated and control series. Prior to the first week, treatment had been severe, especially in barley, and in barley the rate of transpiration did not recover to the level of the control. For the seventh

week, neither rye nor barley recovered to the level of the controls. On other occasions—the fourth and tenth weeks—the rates equalled or exceeded the

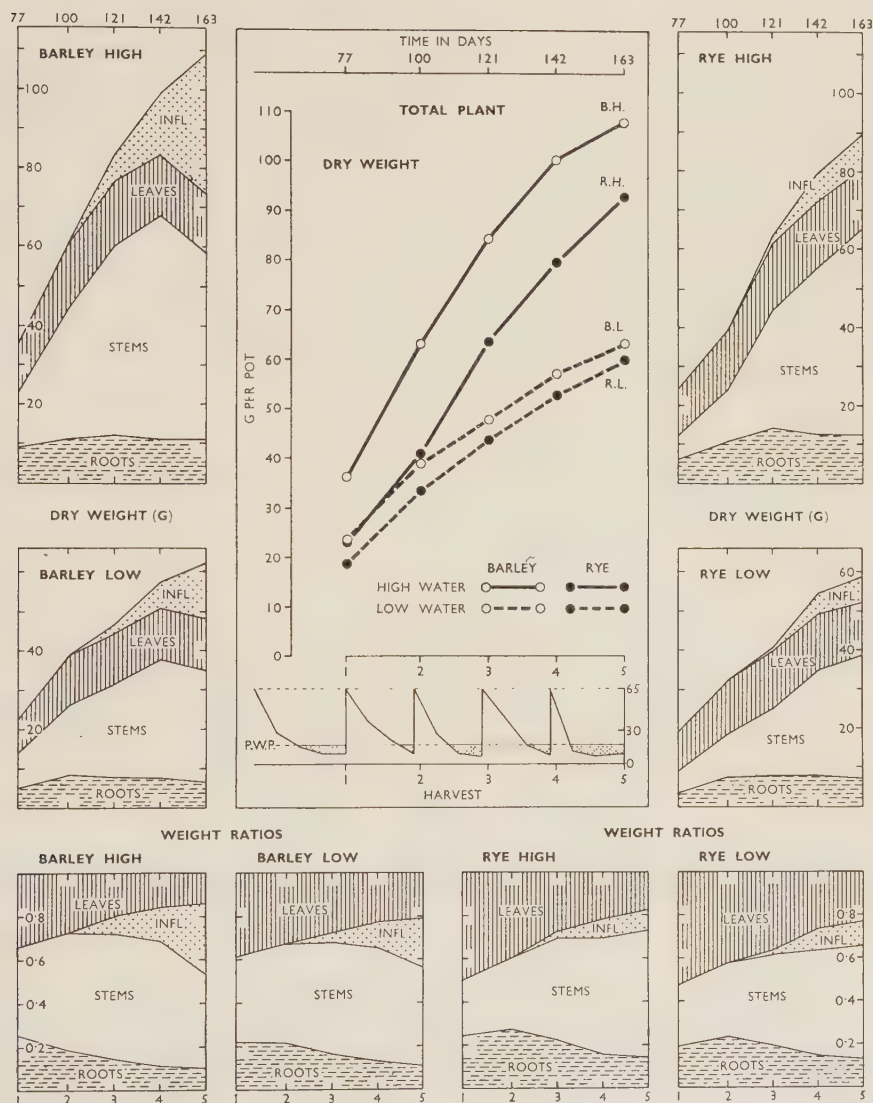


Fig. 2.—Growth (dry weight) of barley and rye as affected by low- and high-water treatments. The marginal figures are summation diagrams of dry matter in roots, stems, leaves, and inflorescences for each treatment separately. At the foot of the page are similar diagrams showing the relative distribution of dry matter between the same plant parts.

rates for the controls, and these occasions were preceded by rather mild low-water treatment.

The fluctuations in the transpiration rates for the controls show a reasonably close agreement with the fluctuations in rate of evaporation from a free water surface.

(c) *Transpiration Ratio*

The transpiration ratio is defined as the ratio of the amount of water transpired to the amount of dry matter formed during the whole or any part of the life-cycle of a plant. Ballard (1933) and Williams (1935) were able to interpret the depressing effects of increased supplies of nitrogen and phosphorus by analysing transpiration ratio into a growth factor and a rate factor, and it is of interest to attempt an interpretation of the specific and treatment differences in the transpiration ratios of the present experiment. The values of Table 2 are based on dry weights for the whole plant including the roots, and are for those portions of the life-cycle ending at the harvests indicated in the table. There are significant interactions between time and treatment class, but these may be neglected for our present purposes.

TABLE 2
TRANSPIRATION RATIOS FOR BARLEY AND RYE SUBJECTED TO HIGH- AND LOW-WATER TREATMENTS

Harvest	Barley		Rye	
	High	Low	High	Low
1	177	171	204	182
2	166	153	188	155
3	164	157	179	160
4	176	158	184	159
5	198	183	211	176

Minimum differences for significance between means ($P = 0.05$) 10.2, ($P = 0.01$) 13.6, ($P = 0.001$) 17.6.

In the control series, the ratio is higher for rye than for barley, and, in the low-water series, rye and barley have virtually the same ratios. Taken together, these effects imply that the transpiration ratio is reduced more by low-water treatment in rye than it is in barley. Looking first at the species difference in the controls, it will be seen that the growth factor would bring about a slight reduction in the ratio for rye as compared with barley. This is so because, if dry matter production is expressed as a percentage of final yield, the curve for barley is always above that for rye. It has also been shown that the transpiration rate per unit weight of leaf tends to be lower in rye than in barley. However, the rate factor of Ballard's original analysis is the somewhat artificial one of rate per unit weight of plant, and he analysed it further as the product of the transpiration rate per unit weight of leaf and the leaf weight ratio (the

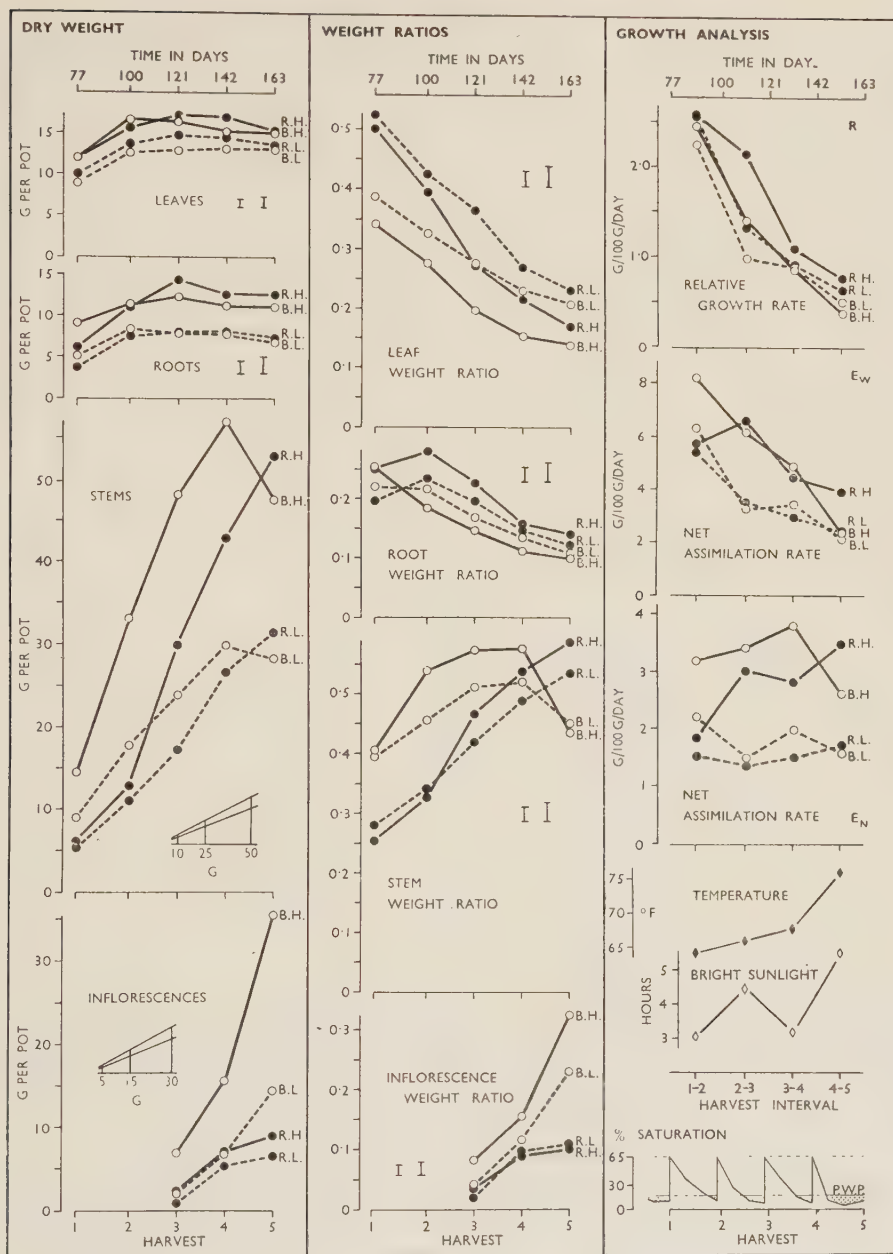


Fig. 3.—Growth (dry weight) curves for the leaves, roots, stems, and inflorescences of barley and rye, weight ratios for these plant parts, and derived data (growth analysis) for the experiment. Where applicable, minimum significant differences are indicated at both 5 per cent. and 1 per cent. levels of significance. For stem and inflorescence dry weights, logarithmic transformations were used, and appropriate minimum significant differences can be read from the inset diagrams. Mean maximum glasshouse temperatures and mean hours of bright sunshine are shown for the four harvest intervals.

ratio of leaf weight to total plant weight). For the present experiment, leaf weight ratios are presented as part of Figure 2. At any one time, the ratio is considerably higher for rye than for barley. That transpiration ratios are higher for rye than for barley may therefore be ascribed to the fact that rye is a more leafy plant than barley.

The reductions in transpiration ratio with low-water treatment are brought about primarily by the reductions in transpiration rate per unit weight of leaf, though the extent of the reductions is modified by the other factors. The growth factor is virtually identical for the low- and high-water treatments of barley, but in rye it would tend to give a higher transpiration ratio with low-

TABLE 3
TOTAL DRY WEIGHT (G PER POT) IN BARLEY AND RYE SUBJECTED TO HIGH- AND LOW-WATER TREATMENTS

Harvest	High Water		Low Water	
	Mean	S.E.	Mean	S.E.
Barley				
1	35.54	1.254	22.94	0.791
2	62.28	1.595	38.34	0.783
3	83.42	1.170	46.99	1.042
4	99.44	2.259	56.32	0.720
5	106.87	1.638	62.25	1.382
Rye				
1	22.24	0.778	18.19	0.482
2	40.15	1.555	32.71	1.134
3	62.70	2.416	43.05	1.570
4	78.62	1.364	51.89	1.258
5	91.75	2.620	58.92	0.868

than with high-water treatment. The effect of treatment on leaf weight ratio in both plants is also such that the transpiration ratio would tend to be increased with low-water treatment. This effect, however, is very much greater for barley than for rye, and it is for this reason that the transpiration ratio is less reduced by low-water treatment in barley.

The importance of leafiness, as measured by the leaf weight ratio, as a factor in water usage is worth stressing. These data show two apparent anomalies. Rye, the more drought-resistant species, is more leafy than barley, and low-water treatment gives a more- and not a less-leafy plant than high-water treatment.

(d) Dry Weight

The dry weight data for the whole plant and for leaves, stems, roots, and inflorescences are presented in a number of ways in Figures 2 and 3. Only total dry weights are presented in tabular form (Table 3), these being basic to the whole experiment. Records of water loss from individual pots had been kept,

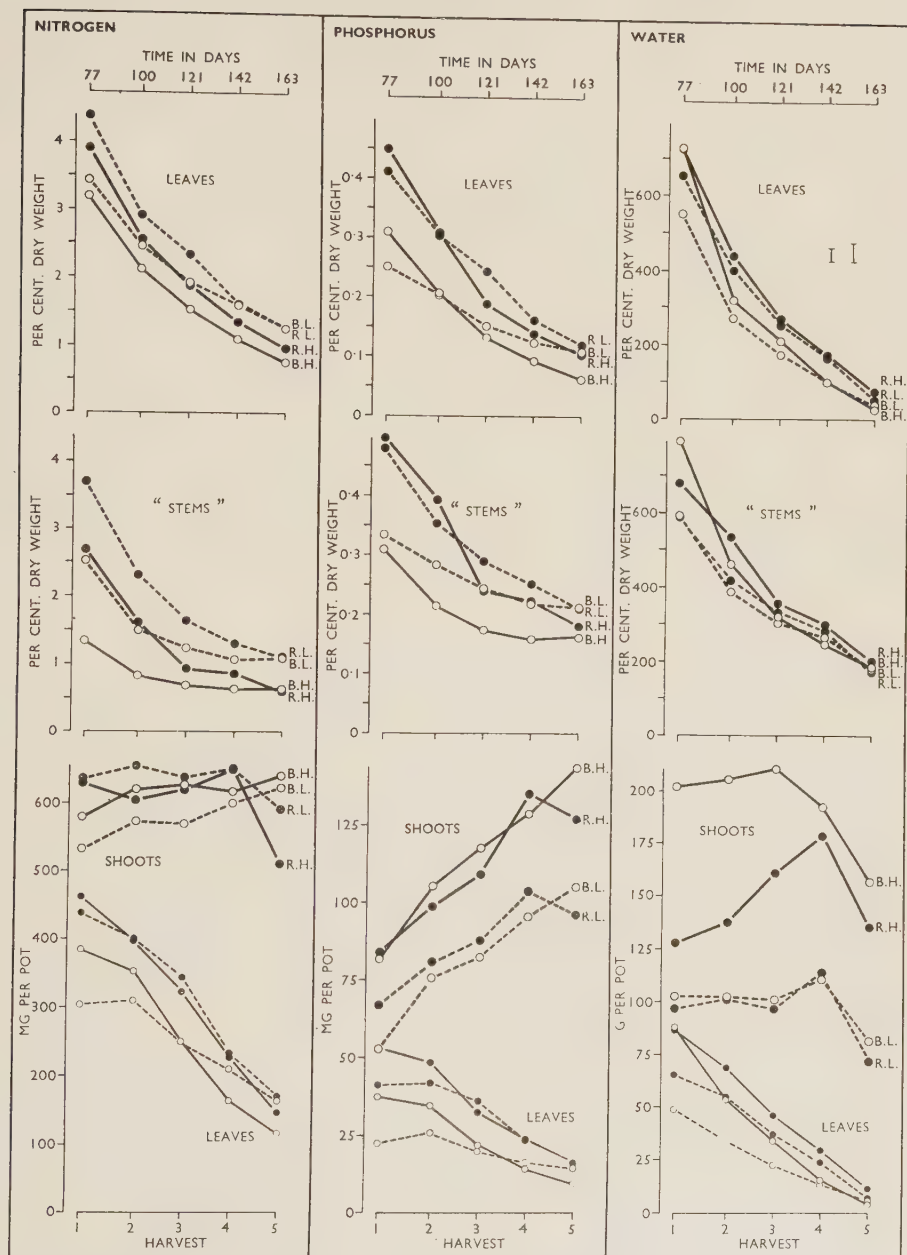


Fig. 4.—Relative and absolute contents of nitrogen, phosphorus, and water in the above-ground parts of barley and rye, as affected by low- and high-water treatments. In this and the succeeding figures, "stems" include the true stems, leaf sheaths, and, when present, inflorescences as well. To aid in distinguishing the absolute data for leaves and shoots, treatment initials have been omitted from the leaf curves.

and it was found possible to use these as ratings for the improvement of the accuracy of mean dry weights (see McIntyre and Williams 1949). The growth curves for the control plants show that vegetative growth was well advanced at harvest 1, the points of inflexion being in the vicinity of harvests 1 and 2 for barley and rye respectively. Leaf and root growth (Fig. 3) were approaching their maxima early in the experiment, and stem growth tends to dominate the picture. Barley has a very much larger inflorescence than rye.

Yield reductions due to low-water treatment were highly significant at all harvests and for both species; even the relatively small effects on rye at harvests 1 and 2 are significant at the 1 per cent. level. Sooner or later the low-water treatment reduced the yields of all plant parts, but the severity of the effect was conditioned by the stage of development of those parts and by the specific differences mentioned above. Stems were more severely affected than leaves, and the inflorescence of barley was more severely affected than that of rye. The effect on root growth was greater than might have been expected from their stage of development at time of treatment, and this effect will be re-examined when considering the weight ratios. The marginal diagrams of Figure 2 present a pictorial version of the distribution of dry matter between leaves, roots, stems, and inflorescences. The upper four diagrams are built up from the absolute weight, and the lower four from the weight ratios.

(e) Weight Ratios

The possession of a relatively large root system is a recognized character of many drought-resistant plants, and it has also been claimed (see Miller 1938 for references) that a typical response of plants to low-water treatment is an increased ratio of roots to shoots. By contrast, the *immediate* effect of low-water treatment in this experiment is to give a significantly smaller root weight ratio with both rye and barley. In barley, however, this effect is reversed after the second period of water shortage, and remains so for the rest of the experiment. In rye, the more drought-resistant plant, the root weight ratio is *reduced* at all five harvests, and significantly so for the first three. A difference between rye and barley which may be relevant here is that, for the controls, the root weight ratio rises to a maximum at harvest 2 in rye, but seems to have passed it already by harvest 1 in barley.

Further differences between barley and rye are shown in leaf and stem weight ratios, high initial leaf weight ratios being balanced by low stem weight ratios in rye. These differences and the subsequent trends in these ratios would seem to be expressions of a delayed developmental pattern in rye. This is also reflected, at least in the controls, in a delayed development of the smaller inflorescence of rye.

Specific differences in response to low-water treatment are also found in leaf and stem weight ratios. Initially both ratios are increased in rye, but significantly so only for the stems. For barley, the initial increase in leaf weight ratio is highly significant, but there is no effect on the stem weight ratio. Examination of the subsequent trends in these ratios suggests that there may

be no essential difference in the pattern of response to low-water treatment, however, and the initial differences could well be associated with the more

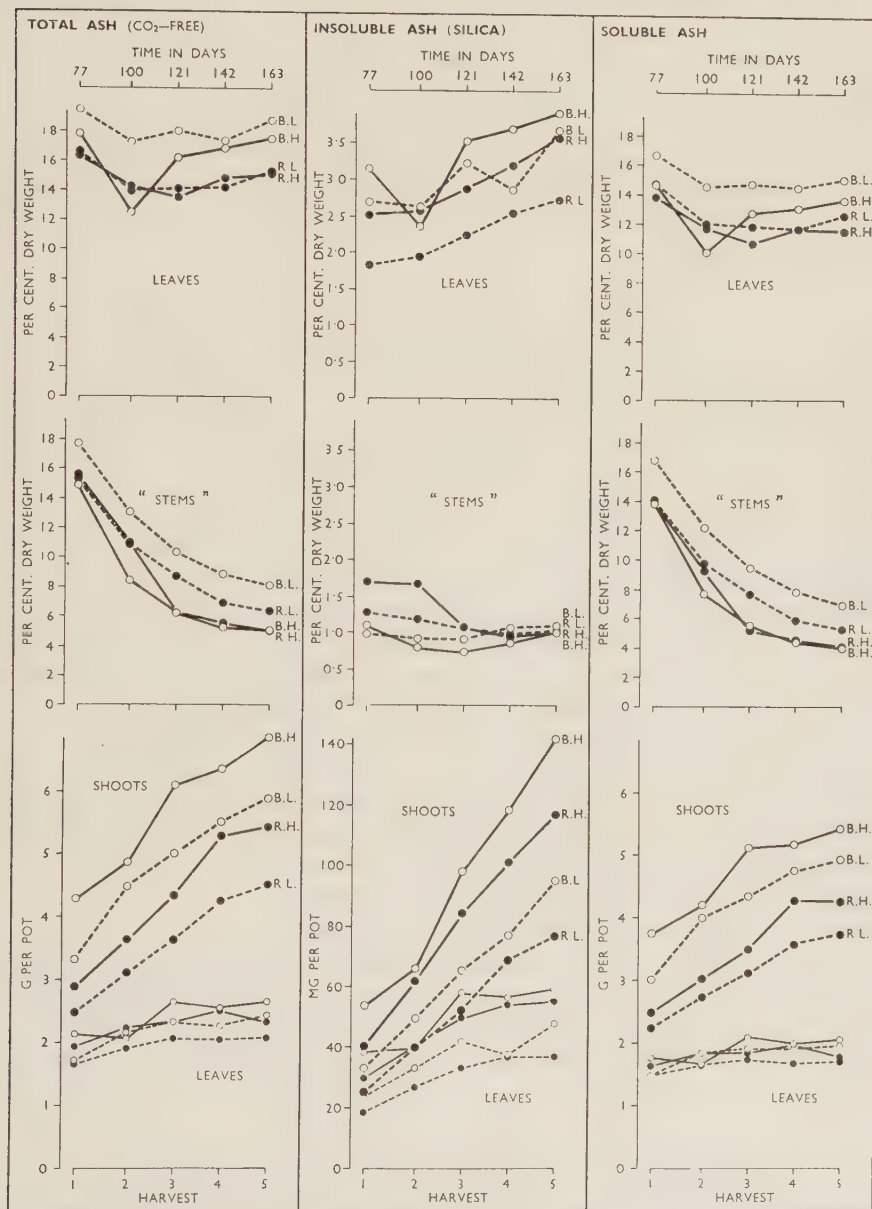


Fig. 5.—Relative and absolute contents of total, insoluble, and soluble ash in the above-ground parts of barley and rye, as affected by low- and high-water treatments.

severe treatment suffered by barley. On the other hand, there remains the curious fact that stem height was quite unaffected by low-water treatment in

rye, but was considerably reduced in barley. Cell elongation was presumably quite unaffected, at least in the primary shoots of rye, by low-water treatment. The number of tillers producing mature heads was considerably reduced by treatment in both barley and rye.

The inflorescence weight ratio was considerably reduced by low-water treatment in barley, but not at all in rye. This fact attests for the drought resistance of rye, and is a result which can scarcely be referred to any difference in severity of treatment. Indeed, rye suffered rather more than did barley during interval 3-4, at which time the inflorescences were growing rapidly. This specific difference in response is well brought out in the four diagrams at the foot of Figure 2.

(f) *Growth Analysis*

Growth rate indices for the experiment are presented in the right-hand panel of Figure 3, and include the relative growth rate R , and net assimilation rates, E_W and E_N , which are based on leaf weight and leaf nitrogen respectively. Mean maximum temperatures for the glasshouse, and mean daily hours of bright sunlight are given for the four harvest intervals, and a soil-water diagram (barley, low water) is repeated at the foot of the page.

No provision was made in this experiment for an initial harvest prior to the first period of treatment. This means that we have no estimate of the effect of this period on the growth indices. However, the much greater reduction in yield in barley at harvest 1 implies a greater reduction in R for this species by low-water treatment. More interest attaches to the values of E_W and E_N , than to those of R , for only in the former can one expect to find any reasonably direct effects of treatment. Moreover, Williams (1946) found that leaf protein was more adequate than leaf weight as an index of the "internal factor" for growth, so that E_N might be expected to be the best available growth index. For the high-water treatment the rising trends in E_N tend to reflect the rising trends in temperature and light; also the depressions in E_N due to low-water treatment follow the severity indices of Table 1 reasonably well. It is a curious fact that E_N tends to be lower for rye than for barley.

The significance of treatment effects can be tested in E_W but not in E_N , and it can be shown that E_W was significantly depressed by low-water treatment for intervals 1-2 and 2-3 in barley, and for interval 2-3 in rye. E_W values tend to converge for later intervals, but there seems no reason to doubt the reality of treatment effects on E_N for intervals 3-4 and 4-5, despite the fact that the significances cannot be assessed.

(g) *Plant Composition and Nutrient Intake*

(i) *General*.—Because of their voluminous character, the data relating to plant composition are presented only in graphical form, but a limited number of sets of numerical data will be available on application to the authors. Statistical treatment is not possible because the material from the five replicates of each harvest class was bulked; however, the sets of data are sufficiently consistent within themselves to justify full discussion of many of the effects.

The data are to be found in Figures 4-7, each figure presenting in columns the values for three distinct plant constituents. At the top of the column in

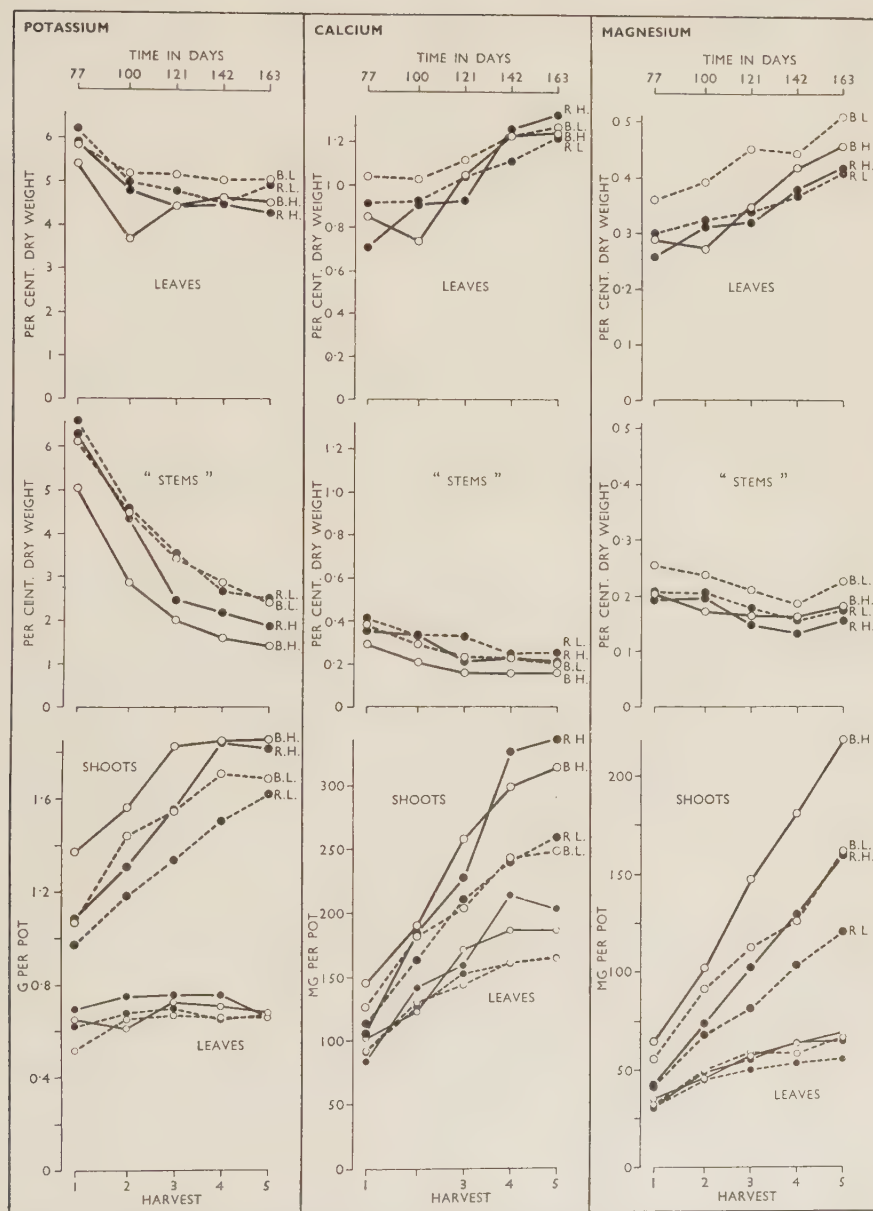


Fig. 6.—Relative and absolute contents of potassium, calcium, and magnesium in the above-ground parts of barley and rye, as affected by low- and high-water treatments.

each case are relative contents for the leaves; then follow relative contents for the "stems". The absolute data are together at the bottom of each column.

It should be remembered that the "stem" fraction of Figures 4-7 includes the inflorescences, the leaf sheaths, and the true stems.

No chemical estimations were made on the roots, so the nearest approach to intake figures is given by the absolute data for the shoots (leaves plus "stems"). Absolute values for the leaves are on the same scale as the shoots, so that "stem" values can be stepped off by difference should these be desired. In most cases there is no confusion between the absolute data for leaves and shoots, but in others the two sets overlap to some extent (especially for sodium, and acid-insoluble ash). Treatment curves are drawn more heavily for the shoots, and treatment initials are included for these but not for the leaf curves.

Nitrogen, phosphorus, and water are presented together in Figure 4 because of several similarities in their trends. Figure 5 presents the trends for total ash, soluble ash, and silica. The first two, which are presented on the same scales, are given for the sake of completeness, though they have little physiological significance. The cations potassium, calcium, and magnesium are together in Figure 6, and possess obvious similarities of trend and treatment effect. Sodium and chlorine also have trends which are linked in a distinctive way (Fig. 7), and manganese is placed with them because sodium and manganese show varying degrees of selective absorption by the plant species under comparison.

In considering the effects of low-water treatment on nutrient intake and relative content, it is necessary to take note of the intake trends for the control series. Nitrogen intake had already ceased in both barley and rye at the time of the first harvest (day 77), but intake was continuous throughout the experiment for silica, magnesium, and possibly also for manganese (data for harvest 5 not available). Shoot intake was also fairly continuous for phosphorus, calcium, sodium, and chlorine. The rate of intake tended to be relatively slower in this group, and there were minor differences between species. Potassium intake ceased rather early in barley but later in rye. Absolute water had attained its maximum at harvest 1 in barley, but rose slowly to harvest 4 in rye.

(ii) *Nitrogen (Fig. 4).*—The relative content of this element for the controls was consistently greater for rye than for barley in the leaves, and in the "stems" it was at first very much greater, but it reduced to equality by harvest 5. These interspecific differences resulted largely from the differences in growth pattern already noted, for the same absolute amount of nitrogen would necessarily be at a higher concentration at any one time in the tissues of the smaller rye plants. Similar considerations apply to the effect of low-water treatment in increasing the nitrogen content of the tissues of both plants, and there is nothing very unexpected in the results.

The absolute data show, however, that the low-water treatment had somewhat delayed the intake of nitrogen by the shoots of barley, and this effect was at first confined to the leaves. That such an effect was slight in the leaves and absent in the shoots of rye, may simply be a reflection of the less severe drought period experienced by rye prior to harvest 1.

There seems, however, to have been a tendency for nitrogen to be excluded from the leaves and to accumulate in the "stems" as a result of wilting,

even though the water content was equally depressed in leaves and "stems" after the soil water had been restored to field capacity prior to harvest 1. It

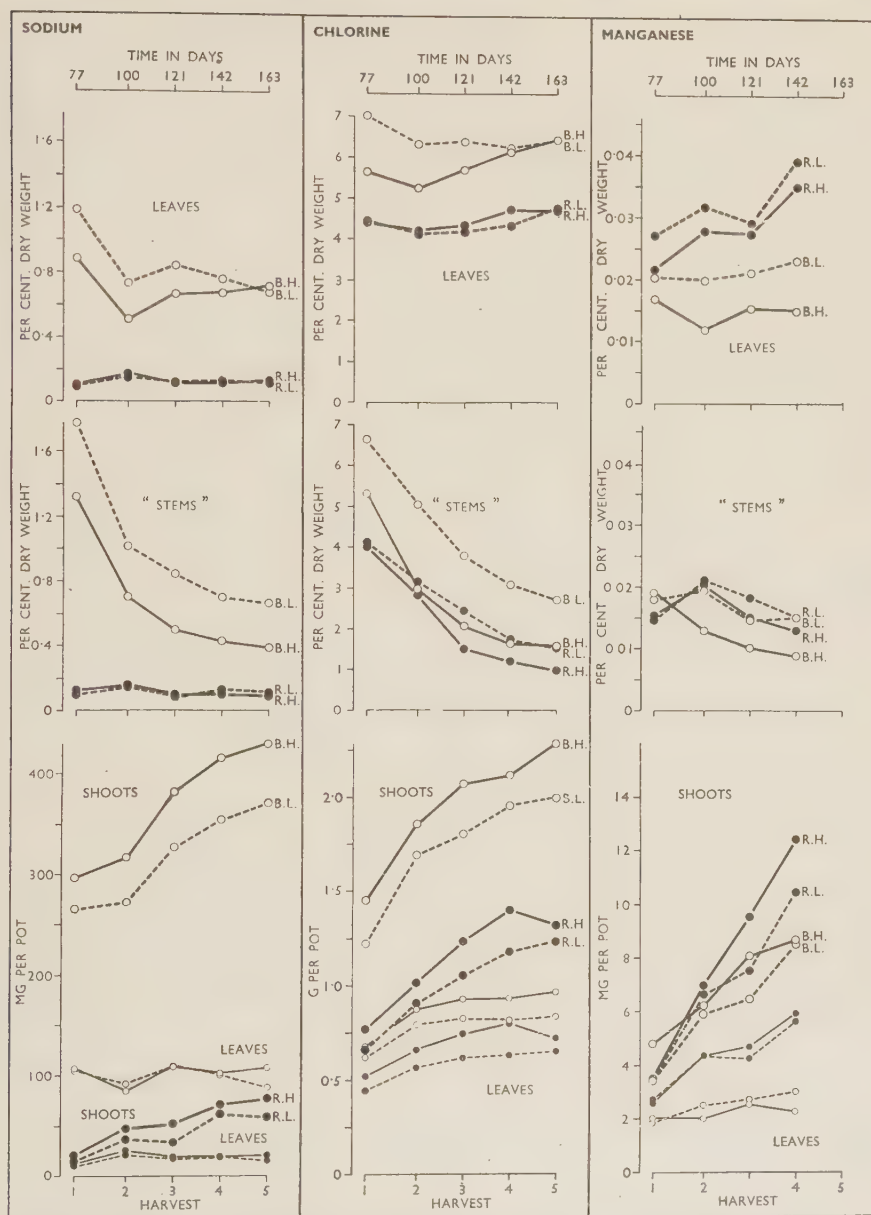


Fig. 7.—Relative and absolute contents of sodium, chlorine, and manganese in the above-ground parts of barley and rye, as affected by low- and high-water treatments.

does not follow that leaf and "stem" water contents were equally depressed during the actual period of wilting, and a fuller understanding of this effect

on nitrogen intake and distribution within the plant calls for a detailed examination of the course of events during the wilting period.

(iii) *Phosphorus* (Fig. 4).—Relative contents of phosphorus for the controls show remarkably similar time trends to those of nitrogen, but early effects of low-water treatment were very different. Instead of an increase there was an initial decrease in the leaf-phosphorus content of both species, and instead of the very big increases in “stem”-nitrogen content there was little initial effect of treatment on “stem” phosphorus. The absolute data show that phosphorus intake by the shoots was considerably reduced by low-water treatment, the greatest effect being in the leaves. The magnitude of this effect is worth stressing. Thus the leaf values, when expressed as a percentage of the control values, are 60 per cent. for barley and 77 per cent. for rye. The corresponding values for nitrogen are 79 per cent. and 95 per cent. respectively.

Nitrogen and phosphorus moved out of the leaves into the “stem” fraction at an increasing rate during the course of the experiment. No such movement of the cations or of chlorine or silica was observed in this experiment.

(iv) *Potassium, Calcium, and Magnesium* (Fig. 6).—Treatment effects on relative content of these three cations are so similar that they may be treated together. However, there are the important differences in intake trends already mentioned for the control series. Thus more than half of the potassium had been accumulated prior to the first harvest by both species, but little more than a quarter of the magnesium had been accumulated in the same period. Calcium is intermediate in this respect. These differences naturally affect the time trends of relative content. Leaf content remained fairly constant for potassium, but rose with time for calcium and magnesium. Potassium content fell rapidly from high initial values in the “stems”. Initial “stem” values for calcium and magnesium, however, were lower than their corresponding leaf values and, though the “stem” values fell at first, they either levelled off or tended to rise during maturation. Similar differences between time trends in potassium and calcium were attributed by Petrie (1934) to the low mobility of calcium and its continued fixation in the tissues. The movement of magnesium within the plant would seem to be governed by similar considerations to those for calcium, though less is known about the forms in which magnesium is chemically fixed in the tissues.

The relative contents of all three cations are consistently increased by low-water treatment in both leaves and stems of barley. Such effects are much less pronounced and somewhat erratic in rye, a difference which may well be due to the difference in severity of treatment. Effects of time and treatment on cation balance (including sodium) will be considered in the discussion.

(v) *Silicon* (Fig. 5).—The element silicon is believed to be absorbed as the silicate ion and to be dehydrated to silica at the cuticular surfaces of the grasses. In the present experiment the time trends of its relative and absolute content are similar to those for magnesium and calcium, but the effects of low-water treatment are quite different. The immediate effect of treatment is a

reduction in the relative content of silica in the tissues, an effect which has a parallel only in the case of phosphorus (Fig. 4). The effect on silica content is erratic in barley but pronounced in rye. It disappears in the "stems" but remains in the leaves of rye as the plants mature.

In view of the very different fates of phosphorus and silicon within the plant, it is difficult to visualize an internal factor which might link these effects. It is perhaps more likely that the low-water treatment reduces the availability of silicate and phosphate ions in the soil, or affects the permeability of the roots with respect to these ions.

(vi) *Sodium and Chlorine* (Fig. 7).—The data for these two elements have many features in common, but the outstanding fact is the strong evidence of selective absorption of sodium as between species. Perhaps the best basis of comparison is that of equivalent percentages (Table 10), from which it will be seen that, at harvest 1, the sodium value is nine times greater for barley than it is for rye. This discrepancy diminishes with time but the difference is still large at harvest 5. Put in another way, the leaves of barley always contained more sodium than the whole of the shoots of rye (Fig. 7). The low equivalent percentages for sodium in rye are balanced mainly by increases in the values for potassium and to a lesser extent by increases in calcium.

Collander (1941) has shown that plants vary greatly in their power to absorb sodium ions when grown in solutions of constant composition. The two cereals, oats and maize, which were included in his study, differed fairly consistently in this respect. Equivalent percentages for sodium were always higher in oats than in maize—in one case nearly seven times higher—but even those for oats were much lower than those reported here for barley.

The relative contents of sodium change rather little with time in rye but fall markedly, especially for the "stems", in barley. The effects of low-water treatment also differ between species, for the relative contents are increased in barley but are little affected in rye.

Time trends and interspecific differences in the relative chlorine contents are very similar to those in sodium for the leaves, but much less so for the "stems". The differences between barley and rye, however, are relatively greater for sodium than for chlorine, and the impression is gained that the chlorine effects follow from and are more or less conditioned by the sodium effects.

(vii) *Manganese* (Fig. 7).—Here again there is evidence of selective absorption, but not to the extent found for sodium. Collander (1941) also found wide variation in his equivalent percentages for this element.

Time trends and treatment effects are similar to those for calcium and magnesium. Rye has taken in relatively more manganese than has barley and this difference tended to widen with time.

(viii) *Water* (Fig. 4).—Water is not usually treated as a plant nutrient, but it is convenient to examine its trends and treatment effects in this place.

The relative water contents show the pronounced downward trends which are to be expected, and the specific and treatment effects are very similar in leaves and "stems".

It is perhaps a little surprising that the very great losses of absolute water from the leaves in all cases did not result in any appreciable loss of potassium from the leaves as predicted by Petrie (1934). The constancy of absolute leaf potassium does not, of course, mean that potassium from old leaves was not re-utilized by younger leaves. This is quite likely, though evidence is lacking in this case.

Watson and Petrie (1940), working with tobacco, found no case in which decrease in absolute leaf-water content preceded net nitrogen export, and this is also true for earlier, less detailed work with gramineous plants by Petrie (1937) and Williams (1938). However, if one may judge from the absolute trends of Figure 4, it does seem possible for the present experiment that net loss of water from the leaves occurred somewhat before net loss of both nitrogen and phosphorus. This may only mean that the nutritional status of the plants was high, so that the demands of developing stems and inflorescences for these nutrients were met to a greater extent from the external medium.

IV. DISCUSSION

(a) *Growth Pattern*

It has often been pointed out that early work on plant response to soil-moisture treatments is of doubtful value because it was based on imperfect knowledge of the physical condition of moisture in soil. This in itself may account for some of the conflicting results which were produced, but confusion has also arisen from a failure to anticipate differences in response traceable to differences in growth pattern of the experimental plants and to the growth stage at which treatment was applied. There has also been a tendency to rely too much on the shoot-root ratio as a single-value index of response to treatment. In what follows, therefore, only those papers which take some account of growth as a factor in response will be considered.

Harris (1914) used nutrient as well as soil-moisture treatments in a study of the ratio of tops to roots in plants. In general, he found this ratio to be decreased by low-water treatment. This result tended to be brought about by a reduction in shoot growth rather than by a direct effect on root growth. In one of his experiments Harris also applied various sequences of low- and high-moisture treatment during three successive stages of growth in wheat. He concluded that his top root ratio was most affected by water treatment during the first of these stages, but his results are difficult to interpret from final yields alone.

Miller and Duley (1925) subjected corn to all possible sequences of "optimum" and "minimum" soil moisture during three successive 30-day periods of growth. Their dry weight data include values for the conclusion of each 30-day period. At the end of the first period, leaf and root weights were greatly reduced, but stem weight was little affected by "minimum" water treatment.

At the end of the second period, "minimum" water during that period also reduced leaf and root weights, but the reduction in stem weight was even greater. At the end of the third period, the main effect of "minimum" water during that period was a reduction in ear weight. There were small but consistent reductions in leaf weight, no effects on stem weight, and a tendency for root weights to be increased. Miller and Duley draw attention to this latter anomaly, but do not attempt an explanation. It is now suggested that where ear development suffers no check, as with "optimum" water, further root growth could have been checked by an intensified competition within the plant for assimilates and mineral nutrients.

It was perhaps to be expected that the application of their "minimum" water treatment at different stages of growth should cause different patterns of response in Miller and Duley's corn plants. A generalization which emerges from the results is that those plant parts which are growing most actively during the period of treatment are those which suffer the greatest check to their growth. The low-water treatments of the present experiments with barley and rye are similar to that of Miller and Duley which received the "minimum" supply during the second and third periods of growth, and the patterns of response are very similar, especially as between barley and corn.

TABLE 4

WEIGHT RATIOS FOR CORN (*ZEA MAYS* L.) AS AFFECTED BY "OPTIMUM" (O) AND "MINIMUM" (M) SOIL-MOISTURE SUPPLY AT THREE SUCCESSIVE 30-DAY PERIODS OF GROWTH*

Weight Ratio	Period 1		Period 2		Period 3	
	O—	M—	—O—	—M—	—O—	—M—
Leaf	0.305	0.341	0.291	0.356	0.306	0.340
Root	0.131	0.137	0.139	0.128	0.114	0.154
Stem	0.263	0.288	0.285	0.266	0.246	0.305
Ear	0.301	0.234	0.285	0.250	0.334	0.201

* Computed from the primary data of Miller and Duley (1925).

Weight ratios for leaf, root, stem, and ear can be calculated from Miller and Duley's data and, since treatment effects are consistent within periods, it is appropriate to present the results (Table 4) as general means at final harvest. Irrespective of when it was applied, "minimum" supply always reduced the ear weight ratio. The effect was greatest for period 3 when the grain was filling, and least for period 2. It is probable that floral initiation took place towards the end of the first period, and the large effect of "minimum" water during this period on ear weight ratio at final harvest may be a reflection of a deleterious effect on floral initiation. The leaf weight ratio was always increased by "minimum" water treatment, but the greatest effect was produced when this was applied during the second period. Root and stem weight ratios were increased

by "minimum" water in the first and third periods but tended to be decreased by this treatment in the second period.

Many of these effects of treatment on the weight ratios of corn (Table 4) are no more than indirect consequences of the fact that those plant parts which are growing most actively at the time suffer the greatest check to their growth. Thus if stem growth is checked severely, leaf and root weight ratios will tend to retain the high values characteristic of the early growth of many plants. It is possible, however, that changes in weight ratios could arise more directly from the action of drought on morphogenetic processes. Indeed, Maximov (1941) has stressed the importance of such effects, particularly those which lead to the formation of the reproductive organs, and quotes at length the findings of Lobov (unpublished data 1939) on the effects of drought on the formation of the spike in wheat. At each successive stage in the development of the spike, drought has its characteristic effect, and this is often not noticeable until long after the drought is over.

TABLE 5

DRY WEIGHT INCREMENTS AND INDICES OF DISTRIBUTION BETWEEN THE LEAVES, ROOTS, STEMS, AND INFLORESCENCES OF FLAX (*LINUM USITATISSIMUM* L.)*

Treatment Interval	Interval (days from sowing)	Water Treat- ment	Total Increment (mg)	Leaves (%)	Roots (%)	Stems (%)	Inflor- escences (%)
Before treatment	64-85	High	212	15	6	79	—
	85-98	High	260	11	5	84	—
First interval of treatment	98-118	High	398	2	1	83	14
	98-118	Low	201	3	-2	82	17
Second interval of treatment	118-136	High	426	-4	4	54	46
	118-136	Low	160	-5	6	56	43

* Computed from the primary data of Tiver (1942).

In the remaining papers to be examined, the level of experimental accuracy permits a more detailed analysis of plant response, and, to this end, distribution indices were computed (Tables 5-9) for relevant harvest intervals of the experiments. Distribution indices are obtained by expressing the increments in dry weight of leaves, roots, stems, etc. for each interval as percentages of the total dry weight increment for that interval. Where such indices are available for a succession of harvests they give quantitative expression to the changing growth pattern, and to the effects of treatment on this pattern. Of special interest is the comparison of indices for the period immediately following the application of low-water treatment.

Tiver (1942) and Tiver and Williams (1943) made parallel studies of growth and development in flax and linseed, and applied a low-water treatment

after flowering. Dry weight increments and indices of distribution are given for flax in Table 5 and for linseed in Table 6. The differences in growth pattern to which Tiver and Williams drew attention are well illustrated by the distribution indices of these tables. Thus, at the time of the first interval of treatment, leaf and root growth had virtually ceased in flax and more than 80 per cent. of the dry matter was going to the stems. With linseed, on the other hand, nearly 30 per cent. was still going to the leaves. The important point for our present purpose, however, is that the low-water treatment had no effect on the distribution indices. It is true that treatment had a rather small effect on the dry weight increment of linseed, but the effect was large for flax. During the second interval of treatment the growth increment was severely reduced in both plants by low-water treatment, yet only in linseed were there any effects on the indices of distribution. Here there were reductions in root and stem indices and rather a large increase in the inflorescence index. The picture gained from the above analysis of weight change and distribution is very dif-

TABLE 6

DRY WEIGHT INCREMENTS AND INDICES OF DISTRIBUTION BETWEEN THE LEAVES, ROOTS, STEMS, AND INFLORESCENCES OF LINSEED (*LINUM USITATISSIMUM* L.)*

Treatment Interval	Interval (days from sowing)	Water Treat- ment	Total Increment (mg)	Leaves (%)	Roots (%)	Stems (%)	Inflor- escences (%)
Before treatment	64-85	High	322	32	13	55	—
	85-98	High	400	23	21	56	—
First interval of treatment	98-115	High	559	27	1	64	8
	98-115	Low	429	28	—1	65	8
Second interval of treatment	115-140	High	1267	—3	16	25	62
	115-140	Low	510	—7	10	12	85

* Computed from the primary data of Tiver and Williams (1943).

ferent from that which might be drawn from the weight ratios in these cases (Tiver and Williams 1943, Table 2, p. 202), for it has been shown for both flax and linseed that low-water treatment *reduces* the inflorescence weight ratio and increases the leaf weight ratio. Once again the effects of treatment on these weight ratios must be interpreted as indirect consequences of the growth pattern of the plant and the time of application of treatment. It would be wrong in this case to infer that the inflorescence got less than its share of assimilates as a result of low-water treatment. In the case of linseed, there is even a suggestion that the inflorescence got more than its share with this treatment.

Petrie and Arthur (1943) subjected tobacco plants to a number of water treatments, and the dry weight increment and distribution values of Table 7 were derived from their data. The tobacco seedlings were transplanted on day

51 and differential watering for the low-water series commenced on day 58. In addition, there were early- and late-temporary-drought treatments. In the first of these (E.D.), differential watering began on day 74 and ended on day 81. The late-drought treatment (L.D.) was begun on day 100 and ended on day 112. The pattern of growth for tobacco is well shown by the distribution indices of the high-water treatment. During the course of the experiment the leaf index fell from 83 to 19, the root index rose from 6 to 34, and the stem index rose from 11 to 47. These changes are associated, at least in part, with the incidence of secondary thickening in roots and stems.

TABLE 7

DRY WEIGHT INCREMENTS AND INDICES OF DISTRIBUTION BETWEEN THE LEAVES, ROOTS, AND STEMS OF TOBACCO (*NICOTIANA TABACUM* L.)*

Treatment Interval	Interval (days from sowing)	Water Treat- ment	Total Increment (g)	Leaves (%)	Roots (%)	Stems (%)
Before treatment	34-41	High	0.047	83	6	11
	41-49	High	0.191	72	4	24
First interval of treatment	49-73	High	7.30	67	13	20
	49-73	Low	3.09	68	12	20
Second interval of treatment	73-85	High	16.41	51	14	35
	73-85	Low	6.04	39	32	29
	73-85	E.D.	14.12	50	17	33
Third interval of treatment	85-99	High	33.6	40	16	44
	85-99	Low	8.2	55	10	35
	85-99	E.D.	32.5	39	15	46
Fourth interval of treatment	99-118†	High	39.8	44	16	40
	99-118†	Low	29.1	48	19	33
	99-118†	L.D.	26.3	32	14	54
Fifth interval of treatment	118†-146†	High	19.9	19	34	47
	118†-146†	Low	16.6	28	25	47
	118†-146†	L.D.	29.8	42	30	28

* Compiled from the original records of an experiment described by Petrie and Arthur (1943).

† Harvesting took more than one day on these occasions.

During the first interval of treatment the dry weight increment was greatly reduced by low-water treatment, but again there was no effect of treatment on the distribution indices. The increments for the second and third intervals of treatment, however, show that the low-water treatment became very severe, so it is not surprising that treatment effects began to appear in the distribution indices as well. The effects are complex and difficult to interpret: the stem index tends always to be depressed by low-water treatment (height growth was

greatly reduced); the leaf index was at first depressed and then increased by treatment; and the root index was much increased and then tended to be depressed by treatment.

The effects of the E.D. treatment (second and third intervals) on dry weight increment are very slight, and it had no effects on the distribution indices. The L.D. treatment (fourth interval), however, had an immediate effect both on the increment and the indices. The leaf index was depressed, the stem index increased, and the root index little affected by treatment. For the final interval, when high-water supply had been restored to the L.D. treatment, these effects were reversed.

TABLE 8

DRY WEIGHT INCREMENTS AND INDICES OF DISTRIBUTION BETWEEN THE LEAVES, ROOTS, AND STEMS OF BARLEY (*HORDEUM VULGARE* L.) AND RYE (*SECALE CEREALE* L.)

Treatment Interval	Interval (days from sowing)	Water Treat- ment	Total Increment (g)	Leaves (%)	Roots (%)	Stems (%)
Barley						
Before harvest 1*	0-77	High	35.5	34	25	41
	0-77	Low	22.9	39	22	39
Second	77-100	High	26.7	18	9	73
	77-100	Low	15.4	24	21	55
Third	100-121	High	21.1	-1	4	97
	100-121	Low	8.7	4	-7	103
Rye						
Before harvest 1*	0-77	High	22.2	50	25	25
	0-77	Low	18.2	52	20	28
Second	77-100	High	17.9	25	31	44
	77-100	Low	14.5	28	29	43
Third	100-121	High	22.6	6	14	80
	100-121	Low	10.3	12	4	84

* Including first interval of treatment.

The data of the present experiment with barley and rye will now be re-examined, and the dry weight increment and distribution indices are to be found in Table 8. Unfortunately, no harvest was taken at the beginning of the low-water treatment, so the values for the period before harvest 1 cannot be taken as a measure of the immediate effect of low-water treatment on dry matter distribution. The distribution indices for this period (0-77 days) are equivalent to weight ratios at harvest 1, and the treatment effects on these ratios are sufficiently small to make it entirely possible that there were no immediate effects of low-water treatment on the distribution indices. With barley for the second interval of treatment, the stem index was greatly depressed, and the indices for roots and leaves were increased by treatment. No such effects were

found with rye for the same interval, and, even admitting that the low-water treatment was less severe for rye, the continued absence of any treatment effect on the stem index during the third interval, does suggest that stem growth in rye was less susceptible to limitation by low-water treatment than it was in barley. Stem height was in fact unaffected by treatment in rye, but was considerably reduced in barley.

TABLE 9

DRY WEIGHT INCREMENTS AND INDICES OF DISTRIBUTION BETWEEN THE LAMINAE, PETIOLES, ROOTS, AND STEMS OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)*

Treatment Interval	Interval (days from sowing)	Water Treatment	Total Incre- ment (g)	Laminae (%)	Petioles (%)	Roots (%)	Stems (%)
First experiment							
Before treatment	38-42	Control	1.20	43	18	13	25
During treatment	42-48	Control	2.43	39	20	13	28
	42-48	Moderate	1.86	31	21	11	37
	42-48	Severe	1.17	28	23	12	37
After treatment	48-55	Control	4.18	30	18	13	39
	48-55	Moderate	3.25	36	16	17	31
	48-55	Severe	2.88	39	17	17	28
Second experiment							
Before treatment	36-40	Control	1.23	40	24	14	22
During treatment	40-48	Control	4.84	41	23	10	26
	40-48	Moderate	3.72	36	23	11	30
	40-48	Severe	2.37	33	24	11	32
After treatment	48-56	Control	5.72	27	21	16	36
	48-56	Moderate	4.45	32	19	14	35
	48-56	Severe	3.72	37	19	15	29

* Computed from the primary data of Gates (1955a).

Gates (1955a, 1955b) has made a careful and detailed study of the effects of wilting treatments of short duration on the growth response of young tomato plants. The wilting treatments were at a moderate and a severe level, but, even at the severe level, soil water did not go below the permanent wilting percentage. From an examination of growth rate and weight ratio data for two closely parallel experiments, Gates concluded that his treatment effects could be summarized as a tendency towards senescence during wilting and a return to a more juvenile condition upon re-watering. For purposes of comparison, these data have been presented as dry weight increments and distribution indices in Table 9. The single day recovery period (day 48-49) was too short to give accurate distribution values, and was united with the following period of each experiment.

From the control values it will be seen that, during the rather brief period considered, the lamina index falls, the stem index rises, and the root and petiole indices change very little. Similarly, only the lamina and stem indices are appreciably affected by the wilting treatments. During wilting, lamina indices were depressed and stem indices were increased, and these effects were reversed during recovery after wilting. Gates found that the pattern of translocation between plant parts had been modified by treatment. Translocation to the upper (younger) laminae was impaired but was continued to the stem, and, upon re-watering, the normal course of translocation was quickly resumed. However, it is not possible from the evidence to judge to what extent the translocation of the wilting period was contributed to by the movement to the stems of breakdown products of hydrolysis in the older laminae. In the overall picture these effects of treatment on translocation tend to cancel out, as seems also to be the case with the L.D. treatment of the tobacco experiment (Table 7).

Taking the evidence as a whole, it is remarkable that there are so many cases in which the immediate effects of low-water treatment on the distribution of dry matter between leaves, roots, and stems are quite small. This could mean that the growth of all rapidly-growing regions tends to be equally inhibited by water stress within the plant, and this interpretation would be in keeping with the claim of Maximov (1941) that drought retards the utilization of carbohydrates (in growth) more than it retards the production of them. If it were otherwise, one might expect that the roots, being furthest from the supply, would tend to get less than their proportionate share of the dry matter produced with low-water supply. This does not seem to be the case, except perhaps at a later stage in rye.

In the light of the foregoing analysis of dry weight increments and distribution indices we may now re-examine the significance of the low-water effects on root weight ratios. It was said earlier that an increased ratio of roots to shoots was more or less accepted as a typical response of plants to low-water treatment. This is not always true and must now be modified. It has been shown that such a response can be brought about as an indirect consequence when the root weight ratio is falling at the time of application of treatment. If, as was certainly the case with rye in the present experiment (Fig. 3), this ratio was rising at the time, the result would be a *decrease* in the root weight ratio with low-water treatment. Such an effect, however, would not explain the continued depression of the root weight ratio in rye, for this ratio later falls with time in both treatments (after day 100), and another cause must be sought. In this connection, it may well be questioned whether Maximov's claim that drought retards the utilization of carbohydrates more than it retards the production of them is always correct. In rye, a drought-resistant plant, the distribution indices for the second and third intervals of treatment (Table 8) indicate that leaf growth is favoured, stem growth is little affected, and root growth becomes adversely affected by continued low-water treatment. While this evidence needs confirmation and is too indirect to be used with any confidence, it could mean for this case that the roots, being furthest from the

supply, do in fact get less than their proportionate share of a limited supply of carbohydrates from the leaves. It is suggested, therefore, that the capacity under water stress of juvenile tissues to continue to operate on a restricted supply of carbohydrates may be characteristic of some drought-resistant plants. Such plants would suffer little permanent damage, and would quickly respond to water when it came. Other characteristics of rye which seem to be in keeping with this picture are the absence, in this experiment, of treatment effects on stem height and on inflorescence weight ratio.

(b) Mineral Nutrition

Wadleigh and Richards (1951) have adequately reviewed the literature on the effects of soil-moisture level on the mineral nutrition of plants and they assert that this is dependent on (i) the extent to which growth and, consequently, mineral utilization might be limited by water supply, (ii) the effect of change in thickness of the moisture films on nutrient availability, and (iii) the effect of variation in soil-moisture tension upon microbiological activity. The evidence was such that these authors found some difficulty in making broad generalizations, but they state that decreasing soil-moisture supply is commonly associated with a definite increase in nitrogen content of the plant tissue, a definite decrease in potassium content, and a variable effect upon content of phosphorus, calcium, and magnesium. The first factor of Wadleigh and Richards' analysis seems to have produced fairly pronounced increases of nitrogen, potassium, calcium, magnesium, sodium, chlorine, and manganese content in both leaves and "stems" of barley. This was also the case for nitrogen and possibly manganese in rye, but increases were small or absent for the other elements. These differences in the effects of low-water treatment as between barley and rye are likely to be due in part to the relatively small initial effect of treatment on growth in rye. The depression in yield increased with time, however, and consistent increases in content of potassium, magnesium, and chlorine appeared in the "stem" fraction of rye with low-water treatment. It will be noted that low-water treatment at no time depressed the potassium content of the tissues of either barley or rye, and this in spite of the fact that the soil-water level was reduced well below the permanent wilting percentage in each case.

Treatment responses with respect to phosphorus and silicon contents in barley and rye differ from those in the other elements, and it has already been suggested that the low-water treatment may have reduced the availability of silicate and phosphate ions in the soil. Wadleigh and Richards have attributed the variable effects of water treatment on phosphorus content to differences in fixing power of the soils used by different workers, the implication being that, for soils with a low fixing power, the growth factor will operate to give increases in phosphorus content. The simplest explanation of the reversal in treatment effect on phosphorus content in the present experiment is that the soil factor was dominant as an initial effect but that this was later reversed by the growth factor. Another possibility which should not be overlooked, however, is that water stress in the tissues and particularly in the leaves may ad-

versely affect the synthesis of organic phosphorus compounds or even cause their hydrolysis. Such an effect might increase the inorganic phosphorus content of the roots sufficiently to retard phosphorus intake. Under the conditions of this experiment re-synthesis would be favoured for some time after each watering and, after the initial setback, net utilization of phosphorus could be at a high rate. Inspection of Figure 4 will show that, after harvest 1, the relative rate of intake of phosphorus by the shoots in the low-water treatments was equal to or greater than that in the controls. Williams (1948) has shown that the balance between synthesis and hydrolysis of nucleic acid is rather easily upset by changing internal factors, and it would not be surprising if it were also sensitive to drastic changes in water stress.

Miller and Duley (1925) give relative phosphorus contents of roots, stalks, leaves, and ears for an experiment with corn, concerning which some aspects have already been considered. Treatment effects vary markedly according to the time of application of their "minimum" water treatment and with past history. Before earing, leaf phosphorus was markedly increased by "minimum" water, and, for two of the three possible comparisons, root phosphorus was also greatly increased. Stem phosphorus was slightly decreased by the same treatment. It is difficult to generalize from the results at maturity, and it is suspected that varying degrees of redistribution of phosphorus within the plant during ear development are responsible for the apparent lack of uniformity of response. The case is quite otherwise with nitrogen, potassium, calcium, and magnesium, for the contents of these elements are, with few exceptions, increased by the "minimum" water treatment. In general, the results for corn are very similar to those for barley in the present experiment.

There seems little reason to doubt that the reductions in tissue content of silica with low-water treatment are due to a reduction in availability with treatment.

(c) Cation Balance

As values are available for the four major cations present in the tissues it is possible to examine the effects of species, water treatment, and time of harvest on the cation balance of the experimental plants. The data were converted to milliequivalents per pot for the shoots as a whole, and the values of Table 10 are percentages of the totals for sodium, potassium, calcium, and magnesium. The very small values for manganese were not included in the totals, the equivalent percentages for this element being based on the totals for the other four elements.

The differences in relative rates of intake of the five elements (see Figs. 6 and 7) are reflected in the equivalent percentages of Table 10. Thus the values for potassium fall about 10 per cent. on the average, but less in the low- than in the high-water series, and less in barley than in rye. The values for calcium, magnesium, and manganese all rise with time, the greatest increase being for magnesium. The sodium values fall in barley but rise with time in rye.

Attention has already been drawn to the very much smaller equivalent percentages of sodium in rye than in barley. The average values are 3.4 and 19.5 per cent. respectively, the difference being balanced by a reverse effect on potassium, and to a lesser extent on calcium. It seems clear that barley has a much greater capacity than has rye to absorb sodium ions when the two are grown on the same medium. It will also be noted that, at harvest 1, which

TABLE 10
EQUIVALENT PERCENTAGES* OF CATIONS IN THE SHOOTS OF BARLEY AND RYE

Treatment	Harvest	Na (%)	K (%)	Ca (%)	Mg (%)	Mn† (%)
Barley, high water	1	21.3	58.1	11.9	8.7	0.29
	2	19.2	55.8	13.3	11.7	0.32
	3	18.8	52.9	14.6	13.7	0.33
	4	19.0	49.7	15.7	15.6	0.33
	5	18.7	47.5	15.8	18.0	—
Barley, low water	1	23.2	54.9	12.8	9.1	0.25
	2	18.1	56.5	13.9	11.5	0.33
	3	19.4	54.0	14.0	12.6	0.32
	4	18.9	53.5	14.9	12.7	0.38
	5	18.9	50.7	14.7	15.7	—
Rye, high water	1	2.3	74.2	14.1	9.3	0.34
	2	4.0	65.9	18.2	11.9	0.50
	3	3.6	64.3	18.5	13.6	0.56
	4	4.0	61.0	21.2	13.8	0.58
	5	4.2	58.2	21.1	16.5	—
Rye, low water	1	1.8	71.9	16.5	9.8	0.37
	2	3.5	66.4	18.0	12.1	0.53
	3	2.7	64.6	20.0	12.7	0.52
	4	4.3	62.4	19.5	13.8	0.62
	5	3.8	62.0	19.4	14.8	—

* For definition see text.

† Expressed as percentage of totals for other four cations.

immediately follows the first period of the low-water treatment, the equivalent percentage of sodium is slightly increased by treatment in barley, and is appreciably lowered in rye. It is tempting to infer from this rather slender evidence that the selective absorption of sodium is actually increased by low-water treatment, and to wonder whether the low uptake of sodium contributes to the drought resistance of rye. Richards and Shih (1940) using statistical methods were able to predict differences in leaf-water content in terms of the contents of potassium, sodium, calcium, and phosphorus. They found large positive correlations between sodium and water contents and a smaller positive effect of phosphorus. The effects of calcium and potassium were small and rather

complex. This work revealed the hitherto unsuspected importance of sodium as a determinant of tissue succulence in plants but it does not of course supply any information on its effect when the same tissues are subjected to water stress. The concentration of sodium ions in wilted tissues could conceivably produce toxic effects leading to the increase in permeability and the intensification of hydrolytic processes that are postulated by Russian workers (Maximov 1941) as major elements in the physiological effects of drought.

This comparative study stresses the need to take into account both the growth pattern of the experimental plant and the stage at which the drought or low-water treatment is experienced. It is believed that many apparent anomalies in the literature on plant-water relations are due to a failure to recognize the relevance of these points. While the original purpose of this study necessitated an interspecific study, it will be abundantly clear that the comparison of drought-resistant and non-resistant varieties of the same species would have been more helpful for the understanding of drought resistance. Such varieties would not necessarily have the same growth pattern, however, and care would be needed to select examples in which this complication was at a minimum.

V. ACKNOWLEDGMENTS

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PHYSIOLOGY OF PEA FRUITS

II. SOLUBLE NITROGENOUS CONSTITUENTS IN THE DEVELOPING FRUIT

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[Manuscript received May 16, 1955]

Summary

Soluble nitrogenous compounds in the seeds and hulls of developing fruits of the pea (*Pisum sativum*, var. Canner's Perfection) were studied at successive stages of growth during two seasons. Of the 26 compounds studied, some were undetectable in some samples and all decreased in the seeds during the period of intense protein synthesis. The results are discussed in conjunction with those of other workers.

I. INTRODUCTION

The experiments described in this paper were designed to identify soluble nitrogenous compounds in pea seeds and hulls at different stages of their development. These analyses were carried out on replicate samples of those used for determinations[†] of the protein and soluble nitrogen, and other constituents (McKee, Robertson, and Lee 1955).

The main observations on the individual compounds represented in the non-protein nitrogen of pea fruits are due to Schulze and Winterstein (1910). They found in immature pods that the hulls contained asparagine (which accounted for about one-half of the total soluble nitrogen), arginine, histidine, tryptophan, leucine, choline, and trigonelline. In the seeds arginine was the main soluble nitrogenous compound, being accompanied by relatively small amounts of asparagine, choline, and trigonelline. Hyde (1953), using chromatographic methods, confirmed the predominance of asparagine in the hulls and of arginine in the seeds of developing peas and listed a number of other compounds. It may be noted that arginine is the chief amino acid liberated on the hydrolysis of protein from pea seeds (Holmes 1953). Snellmann and Danielsson (1953) recorded the presence in developing pea seeds of peptides containing two to six amino acid residues. Auclair and Maltais (1952) reported the occurrence of γ -aminobutyric acid in peas. They also tentatively identified an unknown spot as β -amino-isobutyric acid. Virtanen and Miettinen (1953) identified homoserine in peas and isolated it in crystalline form. Bisset (1954) recorded homoserine, citrulline, and α -aminobutyric acid in immature pea seeds.

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II. MATERIALS AND METHODS

(a) Sampling

The peas (*Pisum sativum*, var. Canner's Perfection) used in the first season (1952) were grown at Boree Cabonne (17 miles west of Orange, N.S.W.). Uniformity of age in the material was obtained by using fruits from flowers tagged on a particular date. Seven samples were collected at 2- to 3-day intervals over a period from 11 to 27 days after tagging. The peas used in the second season (1953) were grown at Hawkesbury Agricultural College, Windsor, N.S.W., and nine samples were collected at 3- to 4-day intervals over a period from 14 to 40 days after tagging.

The seeds were separated from the hulls and known weights were placed in cans containing enough absolute ethanol to make the final concentration approximately 75 per cent.

(b) Separation of Alcohol-Soluble Nitrogenous Compounds

The seeds or hulls were blended in the alcohol for 3 or 5 min respectively in a Waring Blendor. The hulls contained fibrous material which was removed by squeezing the homogenate through muslin. The homogenates were filtered through Whatman No. 31 filter paper, yielding clear, bright green filtrates, containing the constituents soluble in 75 per cent. ethanol.

(c) Preparation of the Extract for Chromatography

In the first season, the method described by McKee and Urbach (1953) was used, except that the elution with sodium hydroxide was omitted. As the concentration of the basic amino acids, especially arginine, was very high in the first season, the samples in the second season were passed through "Zeo-Karb" 216, in its potassium form (Wieland 1944), to separate the basic amino acids. The neutral and acidic amino acids were then passed through "Zeo-Karb" 215 and treated as in the first season. Most of the excess hydrochloric acid was removed from the eluate containing the basic amino acids by six successive vacuum distillations to dryness.

(d) Chromatographic Technique

(i) *Standard Method*.—The ascending technique as described by Wolfson, Cohn, and Devaney (1949) was used. Generally, two-dimensional chromatograms were run, using phenol-water (4:1 w/v) in the first direction and *n*-butanol-acetic acid-water (4:1:1 by volume) in the second direction. The dry chromatograms were sprayed with an 0.1 per cent. solution of ninhydrin in 95 per cent. ethanol.

(ii) *Circular Chromatography*.—It was found that, with the standard two-dimensional method, the basic amino acids formed a streak across the chromatogram, whereas circular chromatography gave good separation. The technique of Zimmermann and Nehring (1951) as quoted by Cramer (1953) was used. Papers were run in phenol-water (4:1 w/v) for 6 hr. *n*-Butanol-acetic acid-water (4:1:1 by volume) was also tried, but the bands were more blurred than those run in phenol-water.

(e) *Specific Tests for Individual Substances*

(i) *Arginine*.—The presence of arginine was confirmed by the modified Sakaguchi reaction of Roche *et al.* (1951). The sodium bromide spray applied immediately after the first spray without drying the paper, gave satisfactory results.

(ii) *γ -Aminobutyric Acid*.—The copper carbonate method of Crumpler and Dent (1949) was used. γ -Aminobutyric acid gave a purple spot.

(iii) *Choline*.—The chromatograms were placed in glass tanks containing iodine vapour (Brante 1949). Some amino acids react with iodine but these had previously been developed with ninhydrin and their positions marked with pencil.

(iv) *Histidine and Tyrosine*.—These amino acids gave reddish pink spots with the modified Pauly reagent of Stepka (1952).

(v) *Homoserine, Pipecolic Acid, and Proline*.—The papers were sprayed with 0.2 per cent. solution of isatin in *n*-butanol, containing 4 per cent. acetic acid (Acher, Fromageot, and Jutisz 1950). The chromatograms were heated for about 1 hr at 50°C and then for 4 hr at 90°C. Homoserine appeared as a pink spot. Methionine sulphone, which overlaps with homoserine, does not give a colour reaction with isatin. Pipecolic acid appeared as a bluish green coloured spot, while proline appeared as a blue spot.

(vi) *The Sulphur-Containing Amino Acids*.—The method of Chargaff, Levine, and Green (1948) was used to detect the sulphur-containing amino acids. Spots appeared in the position of cysteic acid and methionine sulfoxide.

Cystine and cysteine, if present in the extracts, were oxidized during the experimental procedure to cysteic acid. Spots for methionine sulphone and methionine sulfoxide both appeared on the chromatograms. Oxidation of the extract with hydrogen peroxide resulted in a spot appearing in the position of methionine sulfoxide, as well as an increase in the size of the spot in the position of methionine sulphone. Therefore it appears that methionine sulphone and methionine sulfoxide are both present in the pea extracts, but either oxidation product of methionine could be an artifact arising during the preparation of the sample for analysis.

(vii) *Trigonelline*.—The phosphomolybdic acid-stannous chloride reaction of Munier (1951) was used. Trigonelline was observed to give a faint yellow spot with the phosphomolybdic acid reagent. This was reduced to a blue spot with stannous chloride.

(viii) *Tryptophan*.—The reaction with Ehrlich's reagent, *p*-dimethylamino-benzaldehyde (Block 1951), was used.

(ix) *Urea*.—The phenol-sodium hypochlorite method of R. J. Williams as described by Block, Le Strange, and Zweig (1952) was used.

The following amino acids also gave a colour reaction with the phenol-sodium hypochlorite reagent:

Threonine, α -alanine, methionine sulphone, methionine sulfoxide, arginine, serine, asparagine	Pale blue
Glycine, γ -aminobutyric acid, β -alanine, glutamine	Bright blue
Lysine	Blue-green
Histidine	Faint green.

III. RESULTS

The following substances were detected on chromatograms in all samples of both hulls and seeds in both seasons: α -alanine, γ -aminobutyric acid, arginine, aspartic acid, glutamic acid, glycine, histidine, homoserine, "leucine",* methionine, methionine sulphone, methionine sulfoxide, serine, threonine, trigonelline, tyrosine, urea, and valine.

In seeds, pipicolinic acid disappeared at the 27th day in the first season and at the 26th day in the second season. Proline was detectable only in the first three samples (up to 17 days) in the first season and in the first sample (14 days) in the second season. In the second season, β -alanine disappeared after the 29th day. The large concentrations of basic amino acids which were not removed in the first season made glutamine difficult to demonstrate. In the second season, however, glutamine, though clearly present in the early samples, was not detected in the samples from the 26th, 29th, and 32nd days. Phenylalanine was present in all seed samples in both seasons and cysteic acid, undetectable in any samples in the first season, was present in chromatograms up to the 35th day in the second season. Asparagine, detectable in all seed samples in the first season, was undetectable in the samples from 32 and 35 days in the second.

In the hulls, the pattern of detectable amino acids was not the same as that in seeds. Phenylalanine, present in only the first four samples (i.e. up to 17 days) in the first season, was detected only in the samples from 26 and 29 days in the second season. Cysteic acid, undetected in the first season, appeared in chromatograms of the first three samples in the second season. Pipicolinic acid, proline, and β -alanine occurred in the first few samples in the first season but were undetectable in the second. Glutamine was present in all hull samples in both seasons except at the 27th day in the first and at the 26th day in the second. Asparagine was detected in all hull samples except for the first sample (14 days) in the second season. Lysine, undetected in the hulls in the first season, was detectable in all samples in the second.

Choline and tryptophan, found by Schulze and Winterstein (1910) in pea hulls, were not detected in hulls or seeds in either season. β -Amino-isobutyric acid (Auclair and Maltais 1952) and baikiain, ethanolamine, putrescine, and γ -methyleneglutamine, tentatively identified in pea fruits (Hyde 1953) were not detected in hulls or seeds.

In 1953, the relative amounts of the different amino acids were compared by running chromatograms with 1, 5, 10, 20, and 50 μ l of each sample, and noting the minimum amount of sample necessary to give a spot with ninhydrin for a particular amino acid. The results so obtained agreed with the general

* "Leucine" may be a mixture of leucine, norleucine, and isoleucine.

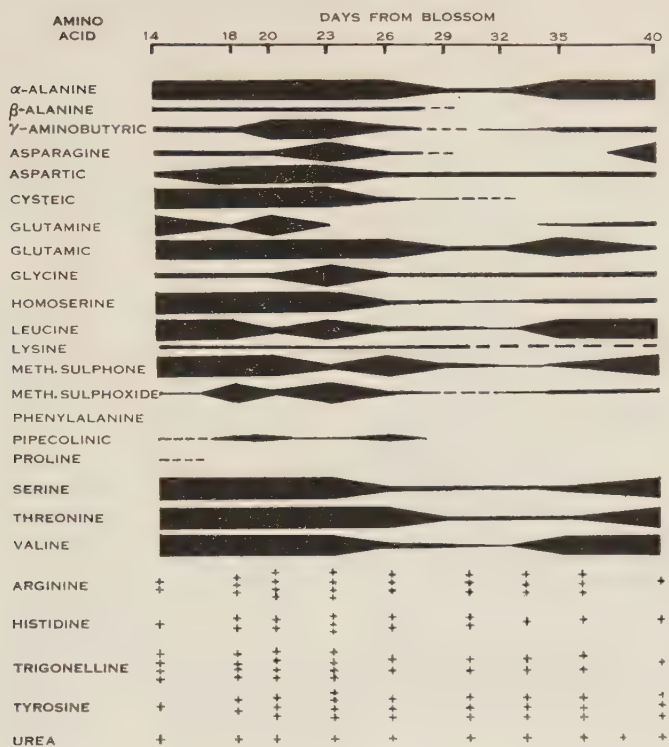


Fig. 1.—Changes in soluble nitrogen compounds in seeds with time from blossom. The width of the lines represents the relative amounts at different times (estimated from the reciprocal of the smallest amount of extract necessary to give a spot). Curves for soluble and protein nitrogen amounts are also shown.

appearance of the developed spots on the papers. By taking the reciprocal of the minimum amount necessary to give a spot, some idea of the relative amounts of the amino acids at different times can be obtained. This has been illustrated diagrammatically by the width of the lines for the different amino acids in Figure 1 for seeds and in Figure 2 for hulls; the width of the line is proportional to the reciprocal of the minimum amount of each sample to give a spot with ninhydrin. An estimate of the changes in arginine, histidine, trigonelline, tyrosine, and urea was obtained by comparison of the sizes of the spots and these are also given in Figures 1 and 2.

Though it must be emphasized that these diagrams cannot be regarded as quantitative, they give a useful indication of the changes in the various amounts of amino acids at different times. While, as has been noted, most of the amino acids occurred in all samples of the seeds and only β -alanine, asparagine, cysteic acid, glutamine, pipercolinic acid, and proline became undetectable at certain times, all the acids decreased in concentration, particularly over the period 23-32 days. Thus in that period α -alanine, β -alanine, γ -aminobutyric acid, asparagine, aspartic acid, cysteic acid, glutamine, glutamic acid, histidine, homoserine, leucine, lysine, the oxidation products of methionine, pipercolinic acid, serine, threonine, trigonelline, and valine all decreased in concentration in the seeds.

In the hulls, β -alanine, asparagine, cysteic acid, and glutamine were undetectable in some samples and other amino acids changed in amount, but there was no consistent change until the time of the last sample when all the amino acids except tyrosine decreased.

IV. DISCUSSION

These results indicate that the changes in the concentration of soluble nitrogen in seeds and hulls, noted by McKee, Robertson, and Lee (1955) in the developing pea fruit, are accompanied by major changes in the individual amino acids. The concentration of soluble nitrogen in the 1953 samples is shown in Figures 1 and 2.

In the seeds, the combined effects of rapidly increasing cell size and increased rate of protein synthesis, with increasing protein concentration, resulted in a decrease in the concentration of soluble nitrogen constituents from about 3.5 mg/g fresh weight at about 20 days to about 1.2 mg/g fresh weight at 26 days and thereafter the soluble nitrogen remained low. About this time, the concentration of all amino acids decreased. Most of these amino acids are known to occur in protein molecules but two amino acids, β -alanine and pipercolinic acid, not known as constituents of protein, were present in the early picks, but not detected later.

The hulls showed a distinct contrast to the seeds. The soluble nitrogen concentration was never as high as in the seeds, and drifted slowly down from about 1.8 mg/g fresh weight on the 14th day to 0.9 mg/g on the 35th. This small change in soluble nitrogen is consistent with the observation that, except for a small rise in absolute amount during the period from 14 to 20 days, the absolute protein nitrogen per hull decreased during the period while, owing to the decreasing fresh weight of the hulls, the protein nitrogen per mg fresh

weight remained approximately constant. Changes in the amino acid pattern were slight in the hulls until the last sample, which corresponded with the biggest change in the soluble nitrogen with time between the 35th and 40th day, when the soluble nitrogen dropped to 0.2 mg/g fresh weight. This simul-

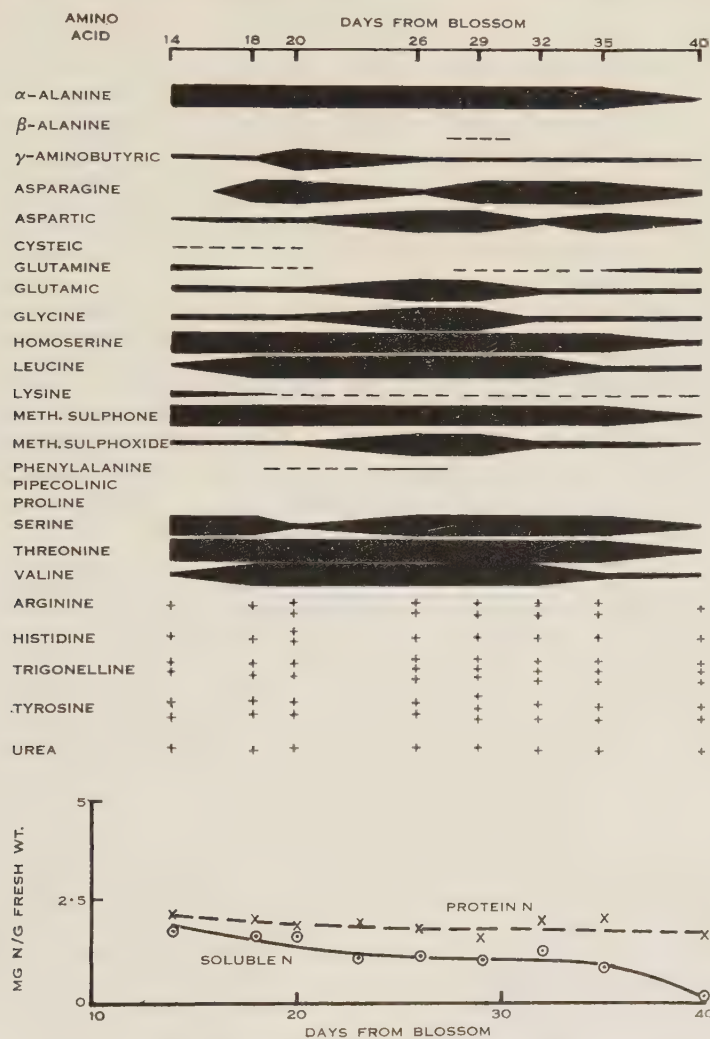


Fig. 2.—Changes in soluble nitrogen compounds in hulls with time from blossom. The width of the lines represents the relative amounts at different times (estimated from the reciprocal of the smallest volume of extract necessary to give a spot). Curves for soluble and protein nitrogen amounts are also given.

taneous decrease in soluble nitrogen and in the individual amino acids, unlike that in the seeds, cannot be attributed to protein synthesis in the hulls since the protein nitrogen per hull decreased over that period from 4.0 to 2.6 mg. The decrease in soluble nitrogen from 1.8 to 0.4 mg per hull must therefore

be attributed largely to export from the hulls. Assuming that the decrease in protein nitrogen was also due to export as soluble nitrogen, the total loss of soluble nitrogen per hull was 2.8 mg. Since each hull contained an average of six seeds, each seed could have gained 0.47 mg. As shown in Figure 1, the soluble nitrogen per seed does not change during the period but the protein nitrogen increases by about 1.8 mg, so about 26 per cent. of the gain in the seed might come from the hull. In this connection it is interesting to note that, while the total soluble nitrogen in the seeds did not increase during the period covered by the last two samples, one-half of the amino acids increased again. Some of these may have been contributed by the "pool" of amino acids in the hulls, which was decreasing simultaneously, but no balance sheet could be suggested on the data available, and full interpretation would require more knowledge than we have at present of the soluble nitrogen compounds which are translocated.

Some differences in occurrence of amino acids in the 1952 and 1953 crops were noted. Unfortunately the semi-quantitative observations on the individual amino acids were not carried out in the first season, but the soluble nitrogen was investigated quantitatively. In the second season the soluble nitrogen per seed reached its maximum on the 23rd day, and in the first season on the 21st day. The soluble nitrogen in the first season did not reach as low a level at the end of sampling as in the second season, though active protein synthesis had begun.

The reduction in concentration of the amino acids during the period of active protein synthesis in the seeds suggests that, for a time, their rate of translocation into the seed or their rate of formation from the translocated precursors was less than their rate of utilization. The complete disappearance of glutamine is interesting in view of the suggestions from work on several plant species that glutamine is used in protein synthesis in preference to the other simple nitrogenous compounds. In the apple, temporary absence of glutamine is one of the few qualitative changes in the amino acid picture as the fertilized ovary develops into the fruit (McKee and Urbach 1953).

The high concentration of soluble nitrogenous compounds in seeds including the pool of amino acids, which was reduced after the initiation of active protein synthesis, suggests that the initial low rate of protein synthesis was not limited by the concentration of the intermediates of nitrogen metabolism. This raises the interesting question of what controls the rate of synthesis which results in the increase after the 23rd day in 1953 and after the 17th day in 1952. It was found that the respiration rate was not correlated with the different rates of synthesis.

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MICROELECTRODE EXPERIMENTS ON *NITELLA*

By N. A. WALKER*

[Manuscript received May 3, 1955]

Summary

Tubular glass microelectrodes containing concentrated KCl solutions have been inserted into internodal cells of *Nitella*, under adequate visual control, and the electric potentials of cytoplasm and vacuole measured. The potential of the cytoplasm is found to be close to that of the sap. For one of the groups of cells studied, this potential was -160 mV referred to the solution bathing the cell when this was $0.0001N$ KCl. Changes in the KCl concentration of this solution caused the potential of the cytoplasm to change in a similar way to that of the sap.

The resting potential difference between the interior and exterior of the *Nitella* cell is thus presumably located at the cytoplasmic outer membrane, as are the potential changes caused by changing KCl concentrations.

The healing process which involves the sealing off of an inserted electrode has been photographed, and simultaneous potential measurements made.

The significance of these results is discussed in the light of present knowledge of the *Nitella* cell.

I. INTRODUCTION

While considerable progress has recently been made towards an understanding of the relation between potential differences and ion concentrations in nerve and muscle, less is known of plant cells. This is so even for *Nitella*, which, like other algae with large coenocytes ("giant cells"), has been extensively studied. The present state of knowledge has been reviewed by Hope and Robertson (1953). Even the individual p.d.'s across the chief cellular membranes, the plasmalemma and tonoplast (i.e. the cytoplasmic outer membrane and the vacuolar membrane, respectively) are not certainly known. This paper represents an attempt to find these quantities.

The p.d. between the vacuole of *Nitella* internodal cells and the medium outside has been measured by Osterhout (1927, 1929, 1930, 1934, 1936, 1949, 1954) under a variety of conditions. He used a method whereby electrical contact with the vacuole was made at a point where the cytoplasm was killed with chloroform. The method is convenient, but open to the objection that the cells do not survive more than a few hours after the experiment begins. It is not capable of measuring the p.d. between cytoplasm and outside medium, and this quantity is of considerable interest if we are to know how the p.d.'s arise, and what their changes (action potentials, concentration effects, potassium effects, etc.) are due to. Various experimenters have also impaled algal cells on

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coarse capillary tubes, but these have again made contact with the vacuole only. In addition, the normal hydrostatic pressure in the cell is released, leaving it in an abnormal state.

Umrath (1930, 1932, 1934, 1953) has made many experiments on *Nitella* internodes using inserted microelectrodes, and is of the opinion that the p.d. he measured is that between the cytoplasm and the outside medium. His results are rather similar to those of Osterhout on the p.d. between sap and outside medium, and for this reason it is important to know whether the tips of his microelectrodes were in fact in the cytoplasm. The evidence for this in Umrath's papers (Umrath 1930, p. 583) is not entirely convincing, as his use of mature, opaque cells, and a dissecting microscope of limited magnification seems to have made direct visual observation of the position of the microelectrode tip very difficult. It is in any case obviously desirable to measure the vacuolar and cytoplasmic potentials in the same cell, or at least in similar ones and under the same conditions, if they are to be compared. It may be noted that while Osterhout used *Nitella flexilis*, Umrath used *N. mucronata* and *N. opaca*.

In experiments described in this paper, young, transparent *Nitella* internodes were used, and the inserted microelectrode tip observed at high magnification ($\times 500$ -1300) in an apparatus of suitable geometry. The location of the tip, either in cytoplasm or sap, could thus easily be distinguished, as seen in the photomicrographs (see Plate 1, Figs. 1 and 2).

The p.d.'s between sap and outside medium, and between cytoplasm and outside medium, were measured on a uniform group of cells of *Nitella* under constant conditions. Often both p.d.'s were measured on the same cell.

It is found that the large p.d. (in these experiments 100-170 mV) between cell interior and outside medium is located at the plasmalemma, the p.d. across the tonoplast being at most a few mV.

The sealing off of an inserted microelectrode, the effects of which on the measured potential were described by Umrath (1932), has been observed and photographed (see Plate 1, Figs. 3 and 4) simultaneously with potential measurements.

In describing the results of these investigations, the arbitrary zero of electric potential is taken as the potential of the solution bathing the cell. Potentials in the cell interior are referred to this zero. Thus in this paper the "potential of the vacuole" is exactly equivalent to the "potential difference between the vacuole and the medium bathing the cell."

II. METHODS

Rooted plants of *Nitella* sp. (probably *N. gloeostachys* A.Br.) were grown in a concrete tank in a warm greenhouse, and in glass pneumatic troughs in the laboratory at 15-25°C. The troughs stood at a north window, but an external blind shaded them from direct sunlight for most of the day. The plants were rooted in a 1-cm layer of sand and garden soil at the bottom of the vessels, which were stocked with snails.

Only young, transparent, internodal cells were used in experiments; they were generally 0.1–0.3 mm in diameter and 3–10 mm long. They were prepared for experiment by cutting off the whorl cells ("leaves"), leaving the apical bud and one basal (mature) internode intact. The preparation was left in 0.0001N KCl solution for about 24 hr after cutting, then mounted on a "Perspex" slide, part of the basal internode being covered with "Vaseline" to secure the preparation in place. A low "Perspex" shelf about 0.4 mm high prevented the experimental cell from moving under the thrust of the microelectrode. This is shown in Figure 1. The "Perspex" slide held 1.2 c.c. of solution bathing the cell, and a steady flow of fresh solution was maintained at 0.3–2 c.c./min as desired. This solution was always 0.0001N KCl, except during experiments on changes of potential caused by changes of external KCl concentration.

Experiments began at least 12 hr after the preparation was mounted on the slide, and continued on each cell for 3–8 days. Cells generally lived indefinitely after the experiments.

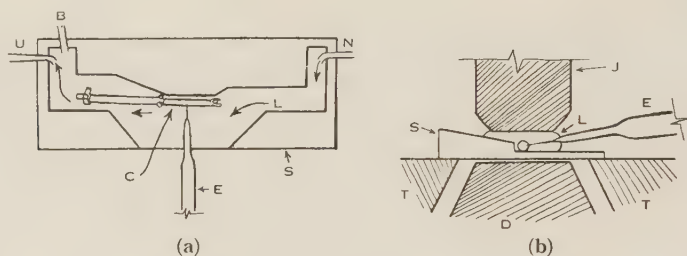


Fig. 1.—(Not to scale). (a) Plan of experimental arrangement. (b) Section. C, *Nitella* cell; E, microelectrode; S, "Perspex" slide; L, bathing solution; N, inflow of fresh solution; U, outflow of solution; B, agar salt bridge; J, microscope objective; T, microscope stage; D, microscope condenser.

The "Perspex" slide was set on the stage of a high-power microscope. The insertion of the microelectrode and the course of the experiment were observed and photographed through a water-immersion objective ($\times 50$) which dipped directly into the bathing solution. A polystyrene collar insulated the objective from the earthed microscope body. A standard low-voltage research lamp was used, fitted with a 3-mm thick heat-filter glass.

Potential differences were measured with an accuracy of ± 3 mV with a valve electrometer. This used a 1S4 valve as an electrometer pentode, operated with low filament, anode, and screen potentials, and biased near the floating grid potential to reduce grid current. The electrometer leads were connected to two symmetrical calomel half-cells. The earthed half-cell was connected to the solution bathing the *Nitella* cell by a 0.1N KCl-agar bridge. The other dipped into the solution filling the microelectrode shank. Experiments indicated that, as expected, the p.d.'s at the KCl-agar junctions were small, and they were therefore neglected. The apparatus was provided with a sufficient electrostatic shield, so that electric pickup effects were negligible.

Microelectrodes of the capillary type were made on a de Fonbrune microforge from "Pyrex" glass tubing of 6 mm outside diameter. While tip diameters of 2-10 μ were used, most were of 3 μ . Tips of smaller diameter than 2 μ were unable to penetrate the cell wall without breaking. An abruptly tapering tip proved to be best, as the hole made in the cell wall was then more effectively sealed. The tips were accordingly drawn in the air stream from the microforge blower.

The shank of the electrode was filled with KCl solution from a pipette, and the solution was forced through to the tip by applying air pressure. The electrode was then used at once, and generally for one insertion only.

In some experiments a microelectrode of 10-20 μ outside diameter was arranged to have a movable glass fibre of 3-5 μ diameter along its axis. The fibre was moved axially by a metal bellows fixed to the micromanipulator holding the electrode. A polythene tube connected the bellows to one of the transmitter pistons of a de Fonbrune micromanipulator. The arrangement is shown in Figure 2. Particles of matter blocking the tip orifice of the microelectrode could be dislodged with the movable plunger.

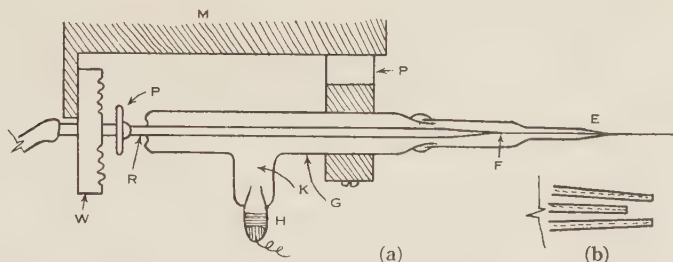


Fig. 2.—(Not to scale). (a) General arrangement of microelectrode with plunger. (b) Tip of microelectrode enlarged. E, Microelectrode; G, "Pyrex" glass T-piece; K, KCl solution; H, calomel half-cell; R, "Pyrex" glass rod drawn down to fibre; F, glass fibre; W, metal bellows; M, micromanipulator; P, polystyrene insulation.

III. RESULTS

(a) Injury of Cell

The insertion of a microelectrode of course injured the cell, but in general there was very little loss of the cell contents, the electrode filling the gap made in the cell wall. Cyclosis nearly always ceased, but often commenced in 1 min, and was normal after 2-4 min. Cells would generally live indefinitely after the experiment, after as many as six or eight insertions, so they are not considered to have been irreversibly injured by an insertion. This is supported by the facts that the potential of the vacuole reached a steady limiting value within a few minutes in most cases, and that generally reproducible results were obtained by successive insertions into the same cell. The method of avoiding injury to the cell when the electrode was withdrawn is described in Section III (c). Re-

sults obtained on cells which died during the experiment were not automatically rejected, but were carefully examined for abnormal features.

(b) Insertions into Cytoplasm and Vacuole

If a shallow insertion of the electrode were made, i.e. to a depth of 20-30 μ , the tip would generally penetrate into the vacuole, but it would be covered by the cytoplasm as soon as cyclosis began. This is clearly shown in Plate 1, Figures 1 and 2, which were taken immediately after the insertion and 3 min later respectively. This type of insertion was used if the potential of the cytoplasm was to be measured. When the potential of the vacuole was required, the electrode was inserted more deeply, i.e. 50-100 μ . In this case, a considerable time, often an hour or more, would elapse before the electrode tip was covered with cytoplasm, and during this period the potential of the vacuole could be measured. No matter to what depth the electrode was inserted, it was eventually covered with cytoplasm, which crept up until it covered the tip. It was not easy to make insertions to a predetermined depth, and it was not found possible to dispense with high-power visual observations of the electrode tip, if cytoplasm and vacuole readings were to be distinguished.

(c) The Healing Reaction and the Sealing Off of an Electrode

If the microelectrode did not completely close the hole made in the cell wall, the loss of cell contents was immediately stopped by the accumulation of debris at the hole. The protoplasm then deposited a layer of refractile substance between itself and the debris. This seems to agree with the description by Nichols (1925) for much larger wounds which took longer to heal. The refractile substance was then laid down over the inserted part of the microelectrode, beginning at the cell wall and progressing inwards until the tip was covered. The electrode was then sealed off from the cytoplasm by this layer, which is visible in Plate 1, Figures 3 and 4. It varied in thickness, being sometimes too thin to be clearly seen (as in Plate 1, Fig. 3) and sometimes much thicker as in Plate 1, Figure 4. By comparing its appearance with that of the cytoplasm and of the glass of the electrode, the refractive index of the sealing substance was estimated at 1.45 to 1.5. Such a refractive index might be attained by, for example, a body consisting of more than 60 per cent. of protein, the remainder being water (extrapolating from Barer and Tkaczyk 1954). A seal of moderate thickness was stiff enough to retain its shape when the electrode was withdrawn, so that no loss of cell contents occurred on withdrawal. This was important in obtaining reproducible results from successive insertions into a single cell. The seal was accordingly always allowed to form before each electrode was withdrawn from the cell.

To anticipate the results reported below, an electrode whose tip was in either cytoplasm or sap recorded a potential of about -160 mV. At about the time when the seal was visibly complete over the electrode tip the magnitude of the recorded potential was fairly suddenly reduced by 30-40 mV, and thereafter remained at the lower value. It was by this reduction of the measured potential and by the subsequent inability of the electrode to record action poten-

tials, that Umrath (1932) detected the sealing process. He referred to it as "die Bildung von Plasmalemma" and investigated the time taken for it to occur, with different solutions in the microelectrode. He may have observed it visually.

In the present experiments the time taken for the seal to be completed varied from a minute to several hours, depending largely upon the depth of insertion of the electrode. Generally it was first deposited around the electrode just inside the cell wall, and then progressively towards the tip: if the tip pro-

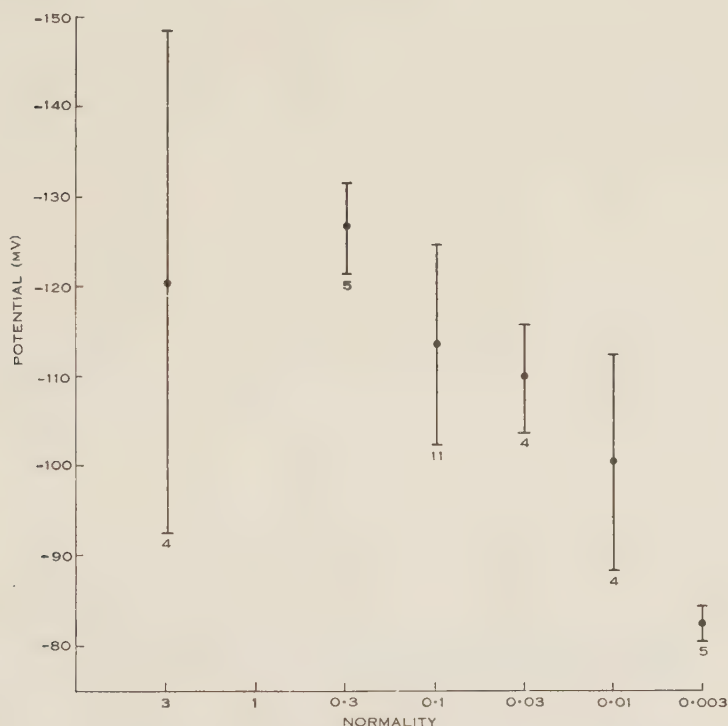


Fig. 3.—Mean potential recorded by an electrode sealed off in the cytoplasm, plotted against the concentration of KCl in the solution filling the electrode. The vertical lines indicate the 95 per cent. confidence limits for the mean of the population sampled: the numerals underneath each point show the number of experiments averaged.

Cell bathed in 0.0001N KCl. (See Plate 1, Figs. 3 and 4.)

jected into the vacuole, cytoplasm would creep up it ahead of the seal, which was, of course, not deposited by the sap. In some shallow insertions, however, the seal seemed to be rapidly deposited around the whole electrode tip simultaneously.

As Umrath observed, there was often considerable fluctuation in the measured potential just before and after the seal was completed. This will be discussed below. After some time, however, the potential settled down to a steady value, which was fairly reproducible, and varied with the concentration of KCl

in the electrode in the manner shown in Figure 3. A comparison of Figures 3 and 4 shows that the mean change in potential upon completion of the seal was close to 35 mV with all the KCl concentrations used in the electrodes (Fig. 3 corresponds with the appearance shown in Plate 1, Figs. 3 and 4; and Fig. 4 with that in Plate 1, Fig. 2).

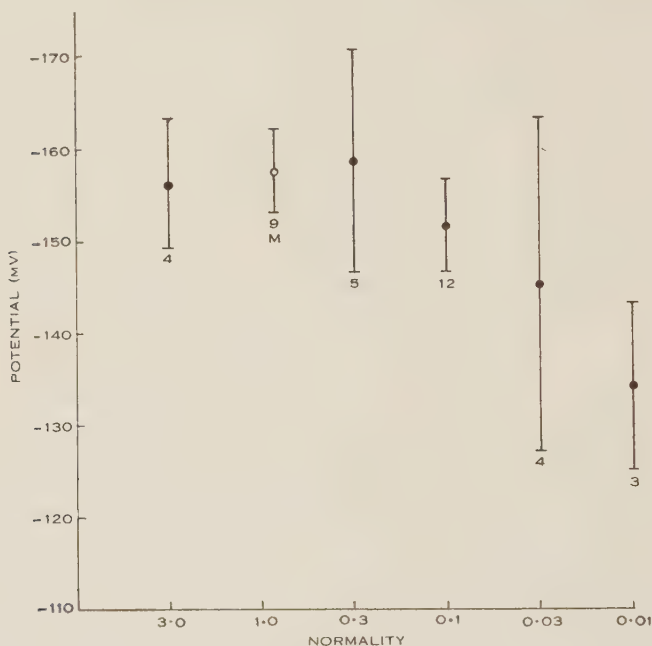


Fig. 4.—Mean potential characteristic of the cytoplasm plotted against the concentration of KCl in the solution filling the electrode. The vertical lines indicate 95 per cent. confidence limits: the numerals show the number of experiments contributing to each mean. The entry marked M is the mean of all the experimental values for 3.0 and 0.3N KCl, and is taken as the mean potential of the cytoplasm. Cell bathed in 0.0001N KCl. (See Plate 1, Fig. 2.)

(d) *The Potential of the Vacuole*

When the tip of the microelectrode was inserted deeply into the vacuole, the recorded potential increased sharply and then slowly, reaching a steady value in a few minutes or less. This value, about -160 mV, was then maintained until the electrode tip was covered with cytoplasm, except that small slow variations sometimes occurred.

The potential recorded by an electrode in the vacuole will approximate to the potential of the vacuole if the junction p.d. between sap and electrode filling is small. Analyses of *Nitella* sap reported by Brooks and Gelfan (1928) and Osterhout (1931) show it to contain approximately 0.1N Cl^- and $0.05\text{--}0.07\text{N}$

K^+ , the balance being largely Na^+ , Ca^{++} , Mg^{++} , with a very small content of organic matter; the pH is about 5. Such a solution may be assumed to have a small junction potential against 0.1N or stronger KCl if no membrane separates the two solutions.

The liquid in the electrode interior was in fact separated from the vacuolar sap by particles of matter which blocked the tip. Upon the insertion of the tubular microelectrode into the cell, the high osmotic pressure in the interior (above 6 atm) drove sap into the tip, which was immediately blocked by particles suspended in the sap. Alternatively, the tip was sometimes blocked by a fragment of cell wall.

To test whether these blockages caused an appreciable membrane potential, several microelectrodes of 10 and 20 μ diameter, fitted with plungers (as in Fig. 2) were inserted into cell vacuoles. After the insertion the plunger could be

TABLE 1
POTENTIAL OF VACUOLE

Concentrations of KCl in Electrode	Mean Vacuole Potential* (mV)	95 Per Cent. Confidence Limits (mV)	Number of Experiments
3N	-160	—	1
0.3N	-160	± 6	5
0.1N	-160	± 6	8
0.03N	-162	± 30	3
0.01N	-157	± 23	3
0.003N	-164	± 12	3
All concentrations	-160	± 4	23

* Cells bathed in 0.0001N KCl solution.

moved forward to dislodge momentarily the matter closing the tip. This produced no changes in the measured potential greater than 3 mV.

In Table 1 are shown the results of a number of experiments in which the microelectrode tip was in the vacuole (as in Plate 1, Fig. 1, but deeper). The microelectrodes were filled with KCl solutions of various concentrations. The lack of any trend in the mean potential as the concentration changes suggests that there is no junction p.d. at the electrode tip with any of the concentrations used; accordingly the results are pooled, and a mean vacuolar potential calculated from them.

These results were obtained in spring and early summer 1954, on a uniform group of 11 cells, all young internodes taken from a single culture vessel. The results on the potential of the cytoplasm described below were obtained from the same group of cells, each cell being used for a number of successive insertions.

(e) The Potential of the Cytoplasm

As mentioned in Section III (*b*) above, a shallow insertion resulted in the tip of the microelectrode being immersed in the flowing cytoplasmic phase (cf. Plate 1, Fig. 2). During the period before the seal was complete, a potential characteristic of the cytoplasm was recorded, and with concentrations of 0.1-3N KCl in the electrode this potential was moderately steady and reproducible. When solutions more dilute than this were used, the seal often formed very rapidly, and even before it appeared complete, transient reductions in the magnitude of the potential were observed. These fluctuations had the same direction and about the same magnitude as the permanent fall of potential due to the seal; they are therefore thought to be due to transient formations of the seal over the electrode tip. Accordingly, in such a case the maximum observed value was taken as the potential characteristic of the cytoplasm.

In Figure 4 the mean potential characteristic of the cytoplasm is plotted against the concentration of KCl in the electrode filling solution. It will be seen

TABLE 2
KCL CONCENTRATION EFFECTS

Potential Measured	Mean Change in Potential* (mV)	Standard Deviation (mV)	Number of Values Averaged
Vacuole	38	8	6
Cytoplasm	35	8	10
Cytoplasm (tip sealed)	37.5	9	14

* As the external KCl concentration is changed from 0.0001N to 0.001N.

that there is a distinct trend in the mean values from -159 mV at 0.3N to -134 mV at 0.01N. It is to be expected that the best approximation to the potential of the cytoplasm will be that recorded by an electrode containing strong KCl, and the agreement between the mean values for 3 and 0.3N KCl supports this. Consequently, the mean of all the determinations using electrodes filled with 3 and 0.3N KCl is taken as the mean potential of the cytoplasm in the cells measured. This mean with its 95 per cent. confidence limits, -158 ± 5 mV, is shown as the entry marked M in Figure 4.

(f) KCl Concentration Effects

Changes in the resting potential of the *Nitella* cell, due to changes in the concentration of KCl solution applied to the outside, are well known, and have been investigated by Osterhout (1929) and others. Osterhout concludes that they are changes in the p.d. across the plasmalemma, because of the rapidity with which the new value of the potential is arrived at. This is shown in the records reproduced in Osterhout (1930).

A series of experiments was made in which the concentration of KCl in the solution bathing the cell was changed while the changes in the potential recorded by an electrode in the sap, the cytoplasm, or sealed in the cytoplasm were observed. The new concentration was more slowly established than in Osterhout's experiments, taking 1-2 min (concentration increasing) or 2-3 min (concentration decreasing).

The results indicate that, as a first approximation, there is no difference between the responses of the potentials of vacuole or cytoplasm (see Table 2).

The method of successive vacuolar and cytoplasmic measurements is unsuitable for investigations of detailed differences between the two. It can, however, be fairly concluded that the main potential changes due to KCl concentration changes occur at the plasmalemma, in agreement with Osterhout's conclusion.

It may also be noted that a sealed-off electrode still records these changes with their full magnitude.

As in Osterhout's experiments, the relation between the cell potential and the logarithm of KCl concentration was found to be roughly linear between 0.0001 and 0.01N. The change in potential for ten-fold change in KCl concentration was smaller than that found by Osterhout for most *Nitella* cells, and more like the values he found for cells leached with distilled water (Osterhout 1949). This may be due to the treatment of the cells used here with 0.0001N KCl.

TABLE 3
SUMMARY OF MAIN NUMERICAL RESULTS

Cell Part Potential*	Mean Value (mV)	95 Per Cent. Confidence Limits (mV)
Potential of vacuole	-160	± 4
Potential of cytoplasm (p.d. across plasmalemma)	-158	± 5
Potential difference across tonoplast	c.0	

* All cells bathed in 0.0001N KCl.

IV. DISCUSSION

The main numerical results are summarized in Table 3.

The quoted 95 per cent. confidence limits are derived from the experimental values: they express the uncertainty of the quoted mean due to random experimental errors and the inherent spread in the potentials of the cells measured. In addition there are possible systematic errors of up to 3 mV due to errors in electrometer calibration, and of the same order due to possible small junction p.d.'s at the microelectrode tip. As these latter will probably be different for cytoplasm and sap, they affect the value of the p.d. across the tonoplast. It is, however, concluded that both the resting potential of the cell, and the potential

changes due to changing ionic environment, have their seat at the plasmalemma, the p.d. across the tonoplast and its changes being of a much smaller order of magnitude. This is established for the *Nitella* species used, under the conditions of measurement; but it is probably also true of this species in its natural environment and for other species of Characeae.

These results do not conflict with those of Osterhout on the vacuolar potential in *N. flexilis*, or with those of Umrath on *N. mucronata*. However, both the steady value of the vacuolar potential, and the time course of the measured potential just after an insertion, as described here, are quite different from those reported by Bennett and Rideal (1954). Their paper is primarily concerned with resistance and capacity measurements, but they mention a measurement of vacuolar potential in which a maximum value was reached immediately after the insertion, the potential then falling in a few minutes to about 18 mV (the sign of the potential is not given). It is not stated whether the potential was measured with the Ag/AgCl electrodes used for the resistance measurements, or what solution bathed the cell; so that a detailed examination of the reasons for the differences cannot be made. However, an Ag/AgCl electrode inserted into the vacuole of a *Nitella* cell (say 0.1N Cl⁻) might be expected to be more negative than a similar electrode in the bathing solution (say 0.001N Cl⁻) by 120 mV (Butler 1951, p. 16) plus the normal vacuolar potential, i.e. by about 300 mV. Bennett and Rideal's result is thus not explained simply on the basis of their use of Ag/AgCl electrodes.

The result reported in the present paper does not provide a basis for deciding which of the various theories of the origin of bioelectric potentials applies to *Nitella*. It is, however, a small part of the body of experimental knowledge which must be built up before such a decision is possible.

The present result does however conflict with the detailed hypothetical scheme of Osterhout (e.g. 1949, p. 555; 1954), as this involves the p.d. across the tonoplast being normally large (equal to the resting potential of the cell) while that across the plasmalemma is normally small. This postulate accounts for the results of his leaching experiments (1934; 1949, p. 552) on the basis that bioelectric potentials in *Nitella* are largely KCl diffusion potentials. In these leaching experiments, the response to externally applied KCl solutions (from which the mobility ratio of K⁺ and Cl⁻ ions can be calculated from diffusion potential theory) was modified drastically by treatment with distilled water, without altering the resting potential (whence the mobility ratio can also be calculated). Osterhout assumes that the resting potential and the potassium response are properties of separate membranes, the tonoplast and plasmalemma respectively, and it is this which now seems untenable. While it is not ruled out, the diffusion potential hypothesis is made more difficult to apply.

It has been suggested that plant cytoplasm contains a high concentration of immobile anions (e.g. Hope and Robertson (1953), and references cited therein), and that a Donnan equilibrium is set up between the medium surrounding the cell and the cytoplasm. The existence of a large p.d. at the outer cytoplasmic surface, under the conditions of the present experiment, is consistent with such a view. In an attempt to obtain confirmation, the equation of

Teorell (1935), which is based on Donnan considerations, might be used to treat the p.d. observed between an electrode containing 0.3N KCl, inserted into the cytoplasm, and the solution (0.0001N KCl) surrounding the cell. If the mobilities of K^+ and Cl^- are assumed equal in cytoplasm, as is nearly true in water, the observed p.d. of -158 mV would lead to a value of about 0.06N for the concentration of immobile anions in the cytoplasm. If this result is then used to predict the p.d.'s which should be observed when other concentrations of KCl are used in the electrode, the agreement with the observed values (Fig. 4) is not good (see Table 4).

Little better agreement with the values given in Figure 4 can be obtained if the mobility of K^+ is assumed less than that of Cl^- (see Table 4); good agreement cannot be obtained for any values of X and u/v . These discrepancies are perhaps not serious, as the mean values in Figure 4 have fairly large confidence limits, and as the exact concentration of KCl in the electrode tip is somewhat uncertain, due to probable diffusion of KCl across the boundary. More serious is the fact that the Teorell equation would require the observed

TABLE 4

COMPARISON BETWEEN OBSERVED POTENTIAL IN CYTOPLASM AND THEORETICAL POTENTIAL CALCULATED FROM TEORELL'S EQUATION

Concentration of KCl in Electrode	Mean Observed Potential in Cytoplasm (Fig. 4) (mV)	Potential (mV) Calculated from Teorell's Equation with Following Values:		
		$X = 0.06N$ $u/v = 1$	$X = 0.095N$ $u/v = \frac{2}{3}$	$X = 0.13N$ $u/v = \frac{1}{2}$
3.0N	-156	-161	-150	-146
0.3N	-158	-158	-158	-158
0.1N	-151	-154	-156	-159
0.03N	-145	-139	-140	-144
0.01N	-134	-115	-115	-119

p.d. to change by 58 mV for each ten-fold change in the concentration of KCl bathing the cell, at the bath concentrations used. Such is not the case for the cells used in these experiments (see Table 2). Osterhout has indeed found this "potassium electrode" behaviour in many *Nitella* cells, but in his leaching experiments has been able to modify this independently of the total p.d. Neither Teorell's equation nor the modified Donnan theory of Vervelde (1953) provide an explanation of these leaching experiments, meeting here the same difficulty as the diffusion potential theory.

As Hope and Robertson have pointed out, we do not know whether there are in plant cells specific ionic pumps, comparable with the sodium pump postulated in nerve fibres. An approach to this problem is the determination of the electrochemical potentials of the various ions in the phases of the system of cell and environment (Ussing 1954). This is approximately achieved if ionic con-

centrations in, and electric potentials of, the various phases can be measured. The present technique has given these potentials for *Nitella*, but ion concentration measurements in the cytoplasm are unfortunately not easy. Work has begun in this Laboratory on the potentials and ion concentrations in the sap of *Nitella* and its environment, as a start on the problem. The concentrations of free ions in the cytoplasm have never been measured satisfactorily, although there are indications that the total concentration is fairly high. Thus Gelfan (1928) found the electric conductivity of *Nitella* cytoplasm to be nearly as high as that of the sap (approx. 0.005 and $0.008 \text{ ohm}^{-1} \text{ cm}^{-1}$ respectively). Bennett and Rideal (1954) quote evidence that the osmotic concentration in the cytoplasm is about equal to that in the sap. A single chemical determination reported by Holm-Jensen, Krogh, and Wartiovaara (1944) suffers from an unknown dilution of the extracted protoplasm with sap: the proportion of ions which are free in solution is also unknown.

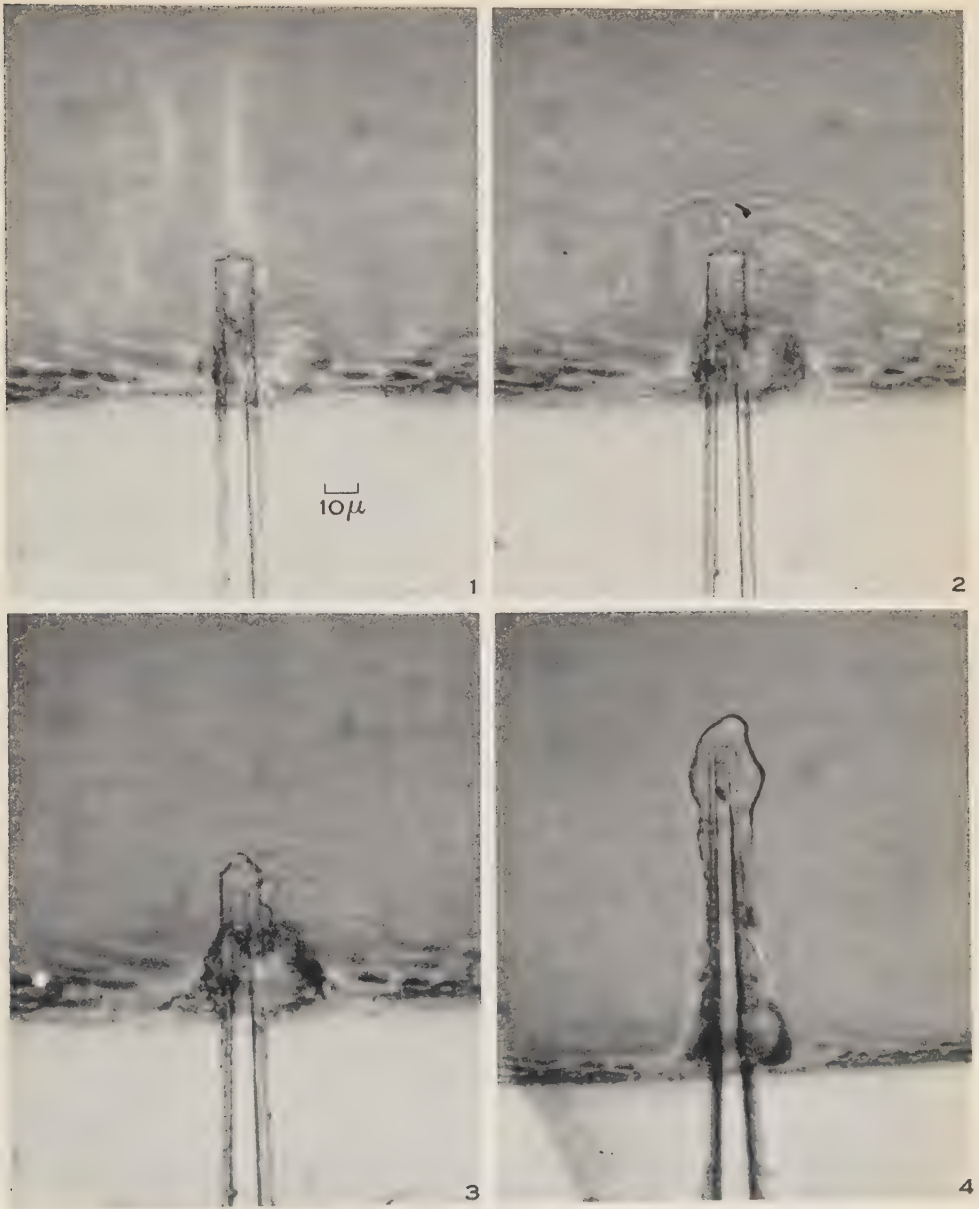
The fall in the measured potential, when an inserted microelectrode is sealed off, was explained by Umrath (1932) as due to a combination of high electric resistance of the seal, and electric leakage along the surface of the microelectrode. If this were so, one would not expect the final potential to reach a reproducible value: and changes in the potential of the cytoplasm would produce smaller changes in the potential of a sealed electrode. Reference to Figure 3 shows that the final potential is moderately reproducible; and Table 2 shows that a sealed electrode reproduces, undiminished, changes in the potential of the cytoplasm. It seems most probable that the fall in observed potential when the seal is complete is due to a p.d. across the seal layer itself.

The nature of the substance forming the seal is unknown. It seems unlikely, however, that it is identical with the outer gel-like layer of cytoplasm ("ectoplasm"—Frey-Wyssling 1948, p. 126), as the seal has a clearly marked interface with the liquid cytoplasm (Plate 1, Figs. 3 and 4). No such interface appears to occur between the gel-like and sol-like cytoplasm in *Nitella*, either visually or in the electronmicrographs of Mercer *et al.* (1955). The seal might consist of a more concentrated cytoplasmic gel, of denatured protein, or of a cell wall substance.

The sealing process is of practical importance, as a factor in all microelectrode experiments on *Nitella*, and probably on other plants as well; Umrath (1932, p. 187) mentions its rapid occurrence in experiments with *Spirogyra* and *Elodea* cells. It should be watched for if its occurrence is not to complicate or invalidate the results of such experiments.

V. ACKNOWLEDGMENTS

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MICROELECTRODE EXPERIMENTS ON *NITELLA*

Photomicrographs of a microelectrode tip inserted into a *Nitella* cell. This tip is 10μ in diameter, i.e. rather larger than those generally used. Magnification $\times c. 500$.

Fig. 1.—Tip in vacuole immediately after insertion. Fig. 2.—Tip covered with cytoplasm, 3 min later. Fig. 3.—Tip covered with barely visible seal, 10 min after insertion. Fig. 4.—Tip-covered with thick seal.

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THE WATER RELATIONS OF GROWTH AND RESPIRATION OF *SALMONELLA ORANIENBURG* AT 30°C

By J. H. B. CHRISTIAN*

[Manuscript received February 7, 1955]

Summary

The influence of water activity (a_w) on growth, respiration, and Na and K content of cells during respiration has been studied for *Salmonella oranienburg*. Sucrose, glucose, glycerol, NaCl, and KCl were added to control a_w . The organism grew in a glucose-inorganic salts medium at 0.97 a_w when any of these solutes was used to adjust a_w , but at 0.96 a_w only when glycerol was employed. Respiration was not inhibited in glycerol-adjusted solutions at a_w 's at which the rate was very low in other solutes. When a_w was controlled by sucrose or glucose, cells oxidizing glucose accumulated K but not Na. Accumulation of K was greatest at 0.975 a_w . In solutions of NaCl, accumulation of K was small and in glycerol solutions it was absent. The differences between glycerol and the other solutes tested are discussed.

I. INTRODUCTION

It has been shown previously (Christian 1955) that the water relations for growth of *Salmonella oranienburg* in a simple, defined medium are similar whether the water activity (a_w) is adjusted by addition of sucrose or by addition of a mixture of three electrolytes. This paper reports the effects of additional a_w -adjusting solutes on growth, respiration, and the changes in internal Na and K levels which accompany respiration.

Other workers have related the respiration rate of bacteria to the concentration of various ions or salts, but no attempt appears to have been made to relate respiration rate to the activity of the solvent.

II. METHODS

The organism was a strain of *S. oranienburg* used previously in nutrition studies (Christian 1955). Growth rates were recorded and the a_w controlled by the methods described by Scott (1953). The a_w 's of glycerol solutions were computed from the data of Scatchard, Hamer, and Wood (1938). The growth medium was a glucose-mineral salts (G.S.) solution (Christian 1955) and the a_w was adjusted to the required levels by addition of sucrose, glucose, glycerol, or a mixture of NaCl, KCl, and Na₂SO₄ in the molal ratio 5 : 3 : 2.

Oxygen uptake was followed in Warburg manometers at 30°C. Cells, grown as described below, were suspended in M/10 KH₂PO₄-Na₂HPO₄ buffer of the desired pH. The contents of the flasks were M/10 glucose (0.1 ml in

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side-arm), cell suspension (1.0 ml), stock solution of a_w -adjusting solute (as required), and glass-distilled water to 3 ml. The centre well contained 0.2 ml of 20 per cent. KOH. The component stock solutions were added by volume, and from a knowledge of their dry weights and densities, the final concentration of each solute was calculated on a molal basis, and hence the total a_w of the mixture determined (Scott 1953). These calculations were checked by isopiestic equilibration and by freezing point determinations.

Readings were taken at 5 min intervals for about 90 min. Oxygen uptake was linear with time and rates were expressed as percentages of the rate at 0.999 a_w . The mean dry wt. of cells per flask was 4 mg.

To study the effect of a_w on the Na and K content of cells of *S. oranienburg*, 250-ml conical flasks containing the same proportions of the constituents used

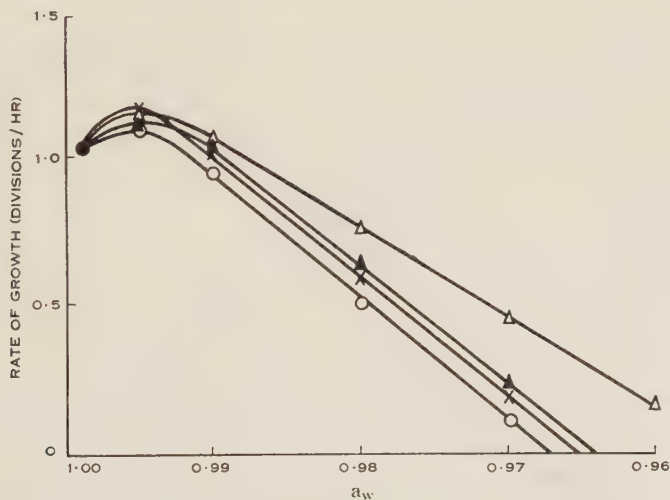


Fig. 1.—Relation between growth rate and a_w for *S. oranienburg* in glucose-salts medium using four methods for controlling a_w .
 ○ NaCl-KCl- Na_2SO_4 mixture in ratio of 5:3:2 moles.
 × Sucrose. ▲ Glucose. △ Glycerol.

in respiration experiments but with a total volume of 15 ml were shaken at 30°C. Duplicate 5 ml samples were taken after 60 min incubation, centrifuged, and the supernatant removed with a capillary pipette. The deposited cells were resuspended in 3 ml of glass-distilled water, steamed for 30 min, and cooled. One ml of N/10 HCl was added and the suspension diluted and analysed for Na and K in an E.E.L. flame photometer. The final concentrations of HCl and of Cl^- ions were below the threshold values for interference with Na and K readings in this instrument (Collins and Polkinhorne 1952). The extraction procedure gave reproducible estimates which were within 5 per cent. of those obtained by ashing the cells. As the cells were not washed prior to analysis, the Na and K values found included contributions from the suspending fluid between the deposited cells. Na and K contents were expressed as μmoles per 100 mg dry wt.

III. RESULTS

(a) Growth

The rates of growth of *S. oranienburg* in G.S. medium in which a_w was controlled by addition of sucrose, glucose, glycerol, and the triple salt mixture are shown in Figure 1. There was no difference at high a_w 's between the rates of growth in media adjusted with sucrose, glucose, or the mixture of salts, but at low a_w 's, the salts were the most inhibitory of these and glucose the least. However, media adjusted with these solutes all supported growth at 0.97 but not at 0.96 a_w . When NaCl and KCl were tested individually as a_w -adjusting solutes the rates of growth at 0.98 a_w were similar to that found with the triple salt mixture, as were the lower limits of a_w supporting growth. Gly-

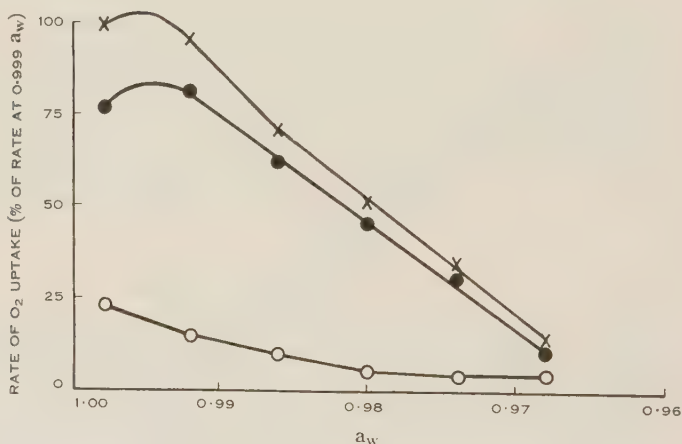


Fig. 2.—Relation between respiration rate and a_w for unwashed suspensions of *S. oranienburg* in which a_w was controlled with added NaCl. × With glucose as substrate (exogenous). ○ Without substrate (endogenous). ● Exogenous rates corrected for endogenous values.

cerol was much less inhibitory than the other solutes at high concentrations and growth was recorded at 0.96 but not at 0.95 a_w . At low a_w 's lag periods were much longer when sucrose, glucose, or glycerol were the adjusting solutes than when the electrolytes were used.

(b) Respiration

(i) *Influence of Growth Medium, Age of Cells, and pH.*—In preliminary experiments respiration rates were compared for cells grown for 6 and 17 hr at 37°C in either nutrient broth (0.999 a_w) or brain-heart-broth (0.993 a_w). Using glucose as substrate comparisons were made in NaCl-adjusted solutions at several a_w 's between 0.999 and 0.97. The general trend of the results was similar for cells grown for both periods in both media although the respiration

of cells grown for 17 hr in brain-heart-broth showed somewhat less inhibition at a_w 's below the optimum. Accordingly cells grown for 17 hr in this medium were used in subsequent experiments.

The rate of oxygen uptake was virtually constant in M/30 phosphate buffer within the initial pH range of 5.8-7.4 in the presence and absence of substrate at 0.999 and 0.98 a_w . In subsequent experiments the initial pH of the controls (0.999 a_w) was 6.9-7.0 and the lowest initial value recorded in any concentrated solution was 6.2.

(ii) *Exogenous and Endogenous Respiration*.—The rate of endogenous respiration of unwashed cells of *S. oranienburg* was 20-25 per cent. of the respiration rate with glucose as substrate at 0.999 a_w . The influence of a_w on

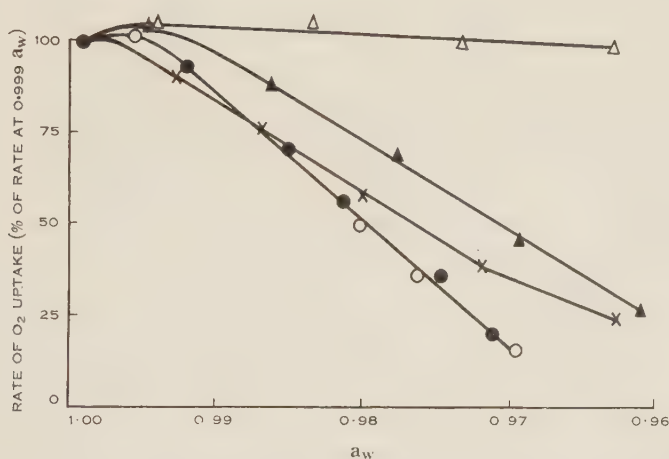


Fig. 3.—Relation between respiration rate and a_w for washed suspensions of *S. oranienburg* using five solutes to control a_w . Substrate: glucose. ○ NaCl. ● KCl. × Sucrose. ▲ Glucose. △ Glycerol.

endogenous and exogenous (glucose) respiration of unwashed cells is shown in Figure 2. Subtraction of endogenous from exogenous rates gave a curve which was a linear function of a_w at a_w 's below 0.992. When cells were washed once in 50 volumes of M/10 phosphate buffer, endogenous respiration fell to less than 5 per cent. of the exogenous rate at 0.999 a_w and exogenous results were similar to those shown in the corrected curve in Figure 2. In later experiments suspensions were washed once in this way and the results were not corrected for endogenous activity.

(iii) *Influence of a_w* .—The relationship between rate of oxygen consumption and a_w for washed cells of *S. oranienburg* is shown in Figure 3. The substrate was glucose and the a_w was adjusted over the range of 0.999-0.96 by addition of the single solutes sucrose, glucose, glycerol, NaCl, and KCl. Re-

sults are the means of at least two experiments. The control rate was always about 400 μl per hr per flask, a Q_{O_2} of 100.

There was no appreciable difference in the rates recorded when NaCl or KCl was the adjusting solute, and the curve was almost identical with that for growth rate against a_w in salt-adjusted synthetic medium when growth was plotted as a percentage of the rate at 0.999 a_w . Glucose and sucrose permitted somewhat higher respiration rates at low a_w 's than did the electrolyte solutions. Respiration rates remained essentially constant as a_w was lowered to 0.96 with glycerol. Glycerol and sucrose were both tested as respiratory substrates for *S. oranienburg*. With sucrose, oxygen uptake was the same as the endogenous rate, and with glycerol the rate was the same as with glucose as substrate both at 0.999 a_w and when adjusted to 0.98 a_w with NaCl.

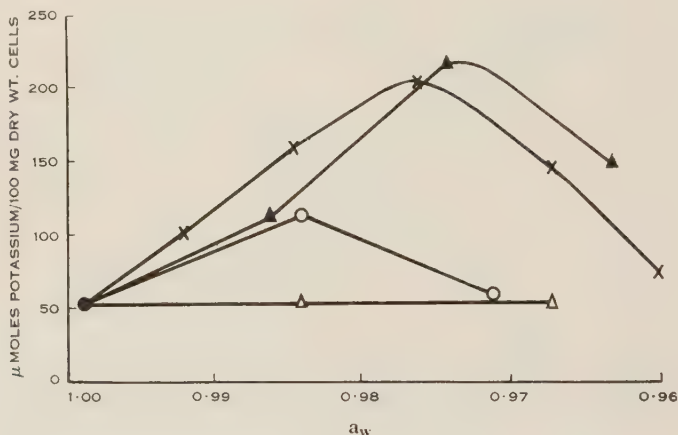


Fig. 4.—Relation between potassium content of cells and a_w for washed suspensions of *S. oranienburg* after 60 min incubation at 30°C using four solutes to control a_w . Substrate: glucose. ○ NaCl. × Sucrose. ▲ Glucose. △ Glycerol.

(iv) *Occurrence of Lag.*—Lag periods of up to 30 min occurred between addition of substrate and commencement of oxygen uptake when a_w was adjusted to low values with NaCl or KCl. Little or no such lag was observed when sucrose, glucose, or glycerol was used.

(c) Na and K Content of Cells

The cells used in the experiments contained initially 34.2 μmoles K and 58.3 μmoles Na per 100 mg dry wt., a K/Na ratio of 0.59. As the molal ratio K/Na of the growth medium, brain-heart-broth, was about 0.1, and that of the washing and suspending buffer about 0.2, these cells, in common with many others, accumulate K in preference to Na.

When cells were washed in buffer and suspended in water no leakage of Na or K was observed over a 3-hr period of shaking at 30°C. As these

results were obtained from analyses of supernatants taken at intervals, they did not exclude the possibility of an immediate change when the cells were first suspended. With glucose as substrate cells incubated at $0.999 a_w$ in phosphate buffer (M/30, pH 6.9) for 60 min showed an increase in K content to $53.7 \mu\text{moles}$ and a fall in Na content to $44.9 \mu\text{moles}$, the K/Na ratio thereby being increased to 1.2. Uptake of K was initially linear with time and gradually approached a maximum after about 90 min. Cells suspended in buffer without substrate showed no change in K or Na contents.

Slight loss of Na was observed after incubation with substrate as a_w was reduced towards 0.96 by addition of sucrose, glucose, glycerol, or KCl, although the loss was usually less than that found at $0.999 a_w$. The high concentrations of Na in the supernatant when a_w was adjusted to low levels with NaCl prevented accurate measurements of the Na content of cells so treated.

The K content of cells incubated with substrate in sucrose- or glucose-adjusted solutions increased rapidly as a_w was reduced to about 0.975 and then fell towards the control value at about 0.96 a_w (Fig. 4). When glycerol was used to adjust a_w the final K content was about the same at all a_w 's tested as in the control at 0.999. An initial increase in K similar to that in glucose-adjusted solutions occurred with NaCl, but this fell again as a_w was reduced to 0.97. Of the non-electrolytes used to adjust a_w , only sucrose contributed significant amounts of K to the incubation mixture. At 0.967 a_w it increased the total K concentration by 5 per cent. K content was not recorded in concentrated KCl solutions.

As the final Na content of cells was about the same at all a_w 's in sucrose-, glucose-, and glycerol-adjusted solutions the shapes of the curves for K/Na ratio, K + Na content, and K content against a_w were similar.

Comparable experiments in M/300 buffer (i.e. one-tenth the external concentrations of Na and K used previously) showed the same trends with the various adjusting solutes. In this dilute buffer, however, an actual loss of K from the cells occurred at $0.999 a_w$. This loss was substrate dependent and a function of time. Similar losses of K occurred in the glycerol-adjusted solutions at all a_w 's. This fall to a new level was apparently related to the very low external K concentration rather than to a_w , since very small increases in the external concentrations of K greatly reduced the loss.

IV. DISCUSSION

When considered in relation to their effects on growth the results of the experiments reported here agree with the conclusions of previous papers (Christian and Scott 1953; Scott 1953; Christian 1955) that the influence of concentrated solutions of normally non-toxic substances is due to their effects on the availability of water.

The respiration data do not conform to such a simple view. For all solutes except glycerol the similarity between the effects of a_w on growth and respiration was close enough to suggest that a_w influences growth mainly through its effect on respiration. The complete absence of respiratory inhibition in

glycerol solutions which markedly inhibited growth does not, however, support such an explanation.

It is, perhaps, more likely that the intracellular loci concerned with growth are different from, or additional to, those concerned only with respiration. Differences in the permeability of membranes at these various loci may then account for some of the observed differences in the effects produced by different solutes. It may be that such differences in permeability are connected with the duration of the observed lag periods. In the measurements of rates of growth the longest lag periods were observed when sugars were the predominant solutes, but the longest lag periods in respiration studies were always associated with electrolyte solutions.

When it occurs, intracellular accumulation of potassium is dependent on respiration, but the greatest accumulation of this cation occurs when the respiration is about one-half the maximum rate. It is difficult to account for this. It is possible that further reduction in respiration rates beyond this point is unable to support a rate of uptake of potassium sufficiently in excess of outward leakage to maintain a high steady state level within the cell. Separate measurements of the inward and outward movement of potassium would clearly be valuable.

Cells immersed in solutions of reduced a_w may reduce their internal a_w to approach osmotic equilibrium with the external solution. Accumulation of solutes or loss of water, or both, would result in such a change. It is of some interest, therefore, to consider the extent to which the observed potassium accumulation may have contributed to such a reduction in the mean intracellular a_w . The maximum accumulation was an increase from 50 to 200 μ moles of K/100 mg dry wt. of cells, when the external a_w was reduced from 0.999 to 0.975. Assuming that the intracellular water is three times the dry wt., this additional 150 μ moles would be dissolved in 300 mg of water. This is equivalent to 0.5M potassium. If it is further assumed that an equal number of univalent anions were accumulated, the reduction in the internal a_w would be about 0.016, which is somewhat less than the 0.024 by which the external a_w was reduced. If, in fact, the internal a_w has followed the external a_w fairly closely it is evident that this would involve either loss of water or accumulation of other solutes.

For cells in glycerol solutions potassium was not accumulated during respiration. It is clear, therefore, that high accumulation of potassium is not vitally concerned in the metabolism of the organism at low a_w 's. Whether or not solutes other than potassium are accumulated from glycerol solutions is a question inviting further study. The penetration into the cell of glycerol itself, or a derivative, may be regarded as likely in view of the high lipid solubility attributed to glycerol. If cells of *S. oranienburg* are, in fact, freely permeable to glycerol, the rise in intracellular glycerol concentration following exposure to concentrated solutions of this substance would increase the concentration of oxidizable substrate available within the cell. Such an increase in substrate concentration may counteract the tendency for respiration to be decreased by the reduced a_w 's. An adequate explanation of the differences between glycerol

and other solutes must, however, await further experiments including studies with other organisms unable to utilize glycerol and with cell-free respiratory systems.

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THE MECHANISM OF THE TRANSMISSION OF POTATO LEAF ROLL VIRUS BY APHIDS

By M. F. DAY*

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Summary

The virus causing potato leaf roll can be recovered from the haemolymph of *Myzus persicae*, the aphid vector. Infective virus has also been separated from the bodies of infected vectors. The virus can be transmitted by an aphid after a moult, and infectivity is retained for at least 8 days; during this time the aphid is able to infect many plants. *M. persicae* is a much more efficient vector than *Macrosiphum euphorbiae*.

Experiments suggest that the virus multiplies to a limited extent in *M. persicae*. When this vector feeds on a plant containing a low concentration of virus there is generally a latent period (of approx. 20 hr at 25°C) between the acquisition feed and a successful inoculation feed. When the vector feeds on a source of high virus concentration occasional transmission is obtained with short (approx. 2 hr) acquisition and inoculation feeds. However, the percentage of transmissions under these conditions with the local strain of *M. persicae* is lower than that reported by American workers. No clonal differences in vector efficiency were found. Attempts to isolate strains of leaf roll virus with different vector relationships were unsuccessful.

The relevance of these results to the mechanism of transmission of viruses by insects is discussed. It is suggested that the occurrence of a latent period in a virus vector is indicative of the passage of the virus from the midgut into the salivary glands via the haemocoel, and the multiplication of the virus in that vector.

I. INTRODUCTION

There are two opposing views on the mechanism of transmission by aphids of the virus causing leaf roll of the potato. Early work (Elze 1927; Smith 1931) demonstrated the existence of a period (the latent period) following a short acquisition feed during which the aphid was incapable of transmitting the virus. The duration of this latent period was estimated at 24-48 hr by Elze, 54 hr by Smith, and 30 hr by Webb, Larson, and Walker (1952). Kassanis (1952) showed that the latent period was variable, but in his experiments it exceeded 49 hr.

On the other hand, several groups of workers have reported transmission of potato leaf roll without a latent period. Loughnane (1943) in Ireland published preliminary results showing transmission following acquisition feeding periods of only 5 min when the inoculation feeding period was 5 days. Kloss-termeyer (1953) in the State of Washington reported transmission following combined acquisition and inoculation feeding periods of 20 min, and a more

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detailed study by Kirkpatrick and Ross (1952) in California demonstrated that potato leaf roll virus could be transmitted by aphids with a minimum latent period of 1½ hr. Larson (personal communication) has transmitted a severe and a mild strain of leaf roll after acquisition and inoculation feeding periods of 1 hr each, but no transmission was obtained when these periods were reduced to 5 min. Finally, MacCarthy (1954) has transmitted leaf roll following a 9½ hr latent period, but demonstrated that a somewhat longer period is usually necessary for transmission.

The discrepancy in the results reported in the previous paragraphs is basic, because it has been suggested (Day and Irzykiewicz 1954) that viruses transmitted following a latent period pass through the haemolymph into the saliva of their vectors, whereas those without a latent period are transmitted mechanically on the mouthparts. The resolution of this problem is therefore crucial to hypotheses concerning the mechanism of transmission of viruses by insects.

There are four possible explanations for the results of Elze, Smith and Kassanis on the one hand, and of Loughnane, Klostermeyer, Larson, and Kirkpatrick and Ross on the other. The early workers all used the potato as the host and as the test plant for work on leaf roll transmission. Later workers used species of *Datura* or *Physalis* in which the virus concentration appears to be greater. It seemed possible, therefore, that differences in virus content of the host plants could have accounted for the differences in the results. Secondly, leaf roll may be transmitted both "mechanically" (without a latent period) and "biologically" (with a latent period) by the same vector. The third possibility is that the two groups of workers were studying different viruses that produced similar symptoms. Finally, the conflicting reports may have been due to the use of strains of aphids differing in their ability to function as vectors.

The experiments reported in this paper were carried out to decide between these possibilities and to elucidate the mechanism of transmission of potato leaf roll virus by the aphid.

II. MATERIALS

Most of the work was done with a colony of *Myzus persicae* (Sulz.) maintained for over four years on *Datura stramonium* L. or *Solanum melongena* L. (egg plant). Some experiments were performed with clones of *M. persicae*. The method of obtaining these is described with the experimental results. A colony of *Macrosiphum euphorbiae* (Thomas), maintained on *D. stramonium*, was also used. All transmission experiments, unless the contrary is stated, were performed with one mature apterous insect per test plant.

The potato leaf roll virus used had been maintained by Dr. E. M. Hutton for several years in Katahdin potatoes in the glasshouse.

The test plants used were *Physalis floridana* Rydb. For some of the work the autotetraploid described by Hutton (1954) was employed. Results with either diploid or tetraploid could generally be assessed within 10 days, but the test plants were generally kept for 14 days after infection. During the winter symptoms developed more slowly and the indicator plants were kept for 3 or 4 weeks. These times are not long enough for root transmission (see Webb,

Larson, and Walker 1952) to have been detected. Very uniform test plants were obtained when the seed was germinated on wet filter paper in petri dishes and planted, 36 to a "flat," soon after the primary root had appeared. The seedlings were generally infected in the two-leaf stage, about 10 days after planting. Transplanting was practised in some early experiments, but the practice was discontinued because of occasional damage to the plants.

The test plants were kept in a conditioned insect-proof glasshouse provided with thermostatically controlled electric heating and evaporative cooling devices. Temperatures were generally maintained between 16 and 26°C during summer and winter, although local heating, due to insolation, often occurred. This glasshouse was regularly fumigated with nicotine and in only two instances were contaminative infections in control plants noted. The results of the experiment in which these occurred were disregarded. The aphid colony was kept, and the infections performed, in separate laboratories.

A total of about 15,000 indicator plants in over 50 different experiments was used in this work.

III. OBSERVATIONS

(a) *Infected P. floridana as a Source of Virus*

A preliminary experiment was performed to determine the optimum time to use infected plants as source plants, and to determine whether infected diploid or tetraploid *P. floridana* or infected potato was the preferable source plant. Eight diploid *P. floridana* and eight tetraploid *P. floridana* were infected with potato leaf roll. At weekly intervals each infected plant was colonized for 4 hr by *M. persicae* that had been starved for 1 hr. These aphids were then placed on test plants, one per plant, and left for 2 days. A young leaf roll infected potato from an infected tuber was similarly used as a source plant. Although it is appreciated that this technique cannot demonstrate small differences in virus concentration of the host plants, it is the only one at present available for potato leaf roll. (Some advantages of the method have been enumerated by Sylvester (1953).) The results (Table 1) demonstrate that the diploid *P. floridana* increased in efficiency as a source of virus until about the third week after infection. Autotetraploid *P. floridana* was a poor source although it was no less attractive to aphids, and epidermal hairs are, if anything, sparser on the leaves of the tetraploid than they are on the leaves of the diploid plant. Hutton (1954) has shown that the autotetraploid reacts more markedly than the diploid *P. floridana*. It is therefore apparent that the efficiency of the plant as a source of virus is not correlated with symptom expression, thus confirming with *Physalis* a conclusion reached by Kassanis (1952) for *Datura tatula*.

A second experiment was carried out to determine the optimum stage to infect diploid *P. floridana* for use as a source plant. The results (Table 2), though based on small numbers, suggest that the infection of plants at about 3 weeks of age is satisfactory for the production of good virus sources. In most subsequent experiments plants of diploid *P. floridana* approximately 3 weeks after infection were generally used as source plants. Subsequent tests showed that diploid *P. floridana* plants remain good sources for at least 7 months after infection.

(b) Details of Transmission of Potato Leaf Roll by Aphids

On the hypothesis of insect transmission of viruses put forward by Day and Irzykiewicz (1954) a number of predictions are possible. If potato leaf

TABLE 1

EFFICIENCY OF VARIOUS INFECTED PLANTS AS SOURCES OF LEAF ROLL VIRUS

Numbers of *P. floricola* infected (out of 36) by single *M. persicae* following acquisition feed of 4 hr and inoculation feed of 48 hr

Time after Infection	Virus Source		
	Diploid <i>P. floricola</i>	Autotetraploid <i>P. floricola</i>	Potato
2 days	0	0	3
1 week	2	1	1
2 weeks	6	1	3
3 weeks	13	0	7
4 weeks	6	3	7

roll is caused by a vector-latent virus, it should be transmitted by the aphid after the latter moults; the virus should occur in the haemolymph of the infected

TABLE 2

EFFICIENCY OF DIPLOID *P. FLORICOLA* INFECTED AT VARIOUS AGES AS SOURCES OF POTATO LEAF ROLL VIRUS

Number of *P. floricola* infected (out of 36) by single *M. persicae* following acquisition feed of 4 hr and inoculation feed of 24 hr

Age of Host at Infection (days)	Time after Infection (days)				
	19	26	34	40	54
13	1	3	1	3	—
16	5	2	0	1	0
22	5	5	2	1	1
24	2	5	0	0	0
30	2	4	0	1	0
35	1	0	3	2	0

aphid; the infective aphid should retain its infectivity for a long period; there should be some degree of vector specificity; and the aphid should be able to acquire the virus from solution. In addition, some of the vector-latent viruses

have been shown to multiply in their insect vectors. These points have been studied and the results are presented in the following paragraphs.

(i) *Transmission by Aphids Following a Moults*.—Elze (1931) and Smith (1931) both reported that the transmission by an aphid of potato leaf roll is not influenced by the intervention of a moult between successive feeds. Confirmation of these reports was sought with the local strain of leaf roll virus. A culture of *M. persicae* was maintained on a potato sprout from a leaf roll infected tuber. Periodically the plant was searched for aphids in the process of moulting. Each moulting aphid was carefully removed to a *P. floridana* indicator plant, and a feeding aphid, as a control, was placed on a similar indicator plant, each plant being covered by a celluloid and muslin cage. The inoculation feeding period was approximately 2 days. Thirty freshly moulted aphids produced 20 infec-

TABLE 3

PERSISTENCE OF POTATO LEAF ROLL VIRUS IN *M. PERSICAE* INFECTIONS (+) FROM SUCCESSIVE TRANSFERS TO *P. FLORIDANA* SEEDLINGS. ACQUISITION FEED 4 DAYS. INOCULATION FEEDS 1½ HR. APHIDS TRANSFERRED OVERNIGHT TO CHINESE CABBAGE

Aphid	Day 1					Day 2					Day 3					Day 4					Total Infections out of 20
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	
1	—	—	+	+	—	—	—	—	+	+	+	—	—	+	—	+	+	—	—	—	8
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0
3	—	—	+	—	—	—	—	+	+	+	—	—	—	+	—	—	+	+	—	+	8
4	—	+	+	—	—	+	+	+	+	+	+	—	+	+	+	+	—	—	+	—	13
5	—	+	+	—	—	—	—	+	+	—	—	—	+	—	+	—	—	—	+	—	7
6	+	—	—	—	—	+	—	—	—	—	+	—	—	—	—	—	—	+	—	+	5

tions, and the 30 normal aphids produced 12 infections. It is apparent that moulting does not render the aphids non-infective and this is strong evidence that the virus is present in the body of the vector and is not carried mechanically on the stylets.

(ii) *Recovery of Virus from the Haemolymph*.—If the virus passes through the midgut of the vector into the haemolymph it should be possible to recover it from that tissue. Haemolymph from leaf roll infected aphids could be drawn into glass micro-injection needles and then injected into normal aphids. Mortality after 24 hr was less than 20 per cent. Satisfactory inoculations were performed on 146 aphids from donors that had fed for 2 weeks on infected potato sprouts. The recipients were immediately placed singly on *P. floridana* indicator plants and left for 2 days. Six infections resulted from these indicators demonstrating that virus could occasionally be recovered from the haemolymph, but that recovery was too infrequent to permit the technique to be used for passing the virus.

(iii) *Retention of Infectivity*.—Experiments showed that single aphids were able to infect a series of indicator plants during a period of several days. In one experiment six aphids were transferred after an acquisition feed of 4 days on a young leaf roll infected potato sprout to *P. floridana* indicator plants at intervals of 1½ hr. Overnight they were placed separately on Chinese cabbage seedlings in which the virus does not multiply. Twenty plants of the indicator were infested by each aphid. One aphid transmitted the disease to 13 of these, two to 8, one to 7, one to 5, and one did not transmit at all (Table 3).

In a second experiment *M. persicae* that had been more than one week on infected potato were placed on a vigorous Chinese cabbage seedling. At intervals aphids were transferred to *P. floridana* indicator plants. Infections were obtained from aphids removed up to the 7th day, but thereafter aphids from the Chinese cabbage were non-infectious. The great majority of original apterae were by that time replaced by their offspring. The length of life of apterae under these conditions was about 10-15 days.

The retention of infectivity was also studied by a third method. In this experiment 36 mature apterous *M. persicae* from an infected potato were placed singly on indicator plants. Every 24 hr for 8 days they were transferred to new indicator seedlings so that the transmission behaviour of each aphid could be evaluated. The temperature approximated 25°C. It might be expected that this method would indicate longer virus retention times than the previous methods, because each insect could possibly reinfect itself when left for 24 hr on a sensitive plant and because the aphids were likely to live longer when they were handled only once a day, than when they were transferred every 1½ hr. Four aphids transmitted the disease the 8th day after they were first placed on the indicator plants.

These experiments demonstrate that *M. persicae* can retain the virus of potato leaf roll for at least a week. It is probable that the virus is retained for the life of the insect (MacCarthy 1954).

(iv) *Vector Specificity*.—Published data on specificity of vectors of potato leaf roll virus have been summarized by Day and Bennetts (1954), and by MacCarthy (1954). The latter considers that only four species of aphids are effective vectors of leaf roll in the field. These are *M. persicae*, *M. ornatus* Laing, *M. circumflexus* (Buckt.), and *M. ascalonicus* (Donc.).

It was planned to repeat with *Macrosiphum euphorbiae* several of the experiments that had been done with *M. persicae*. Under the name of *M. gei*, *M. euphorbiae* had been reported by Smith (1929) and Cottier (1931) to be incapable of transmitting the virus, and early attempts to demonstrate transmission by this species using *P. floridana* were uniformly negative. However, transmission was obtained in our experiments when *Datura tatula* L. was used as source and indicator plants. Even under these conditions transmission was too inefficient for detailed experiments to be completed. For example, after an acquisition feed of 3 days on a *D. tatula* infected for 3 weeks, 5 aphids were placed on each of 9 indicator seedlings. After 4 weeks only 2 of these showed symptoms. It was therefore concluded that, although *M. euphorbiae* could

transmit potato leaf roll, the difficulties of working with it were too great to warrant detailed tests. Similar results reported after the completion of our tests, were obtained by MacCarthy (1954) with *Macrosiphum solanifolii* (Ashm.) which is probably the same species of aphid.

Further information is required for generalizations concerning the vector specificity of potato leaf roll, but it is already apparent that this specificity is greater than would be expected in a vector-direct virus.

(v) *Acquisition from Solution of Virus by the Vector.*—It is known that aphids cannot acquire non-persistent viruses from solution and reasons for this have been suggested by Day and Irzykiewicz (1954). If a virus is ingested and passes from the midgut into the salivary glands via the haemocoel there is no reason why the aphid should not be able to acquire it from solution, although a number of workers have tried to accomplish this without success. The following experiments have been carried out: 44 highly infectious plants of *P. floridana* 3 weeks after infection were ground in a mortar with a little sand. The juice was squeezed through cheese cloth and yielded 21 ml of sap. This was centrifuged for 10 min at 10,000 *g* in a refrigerated centrifuge. The supernatant was then clarified at 100,000 *g* for 45 min in a Spinco ultracentrifuge at 0°C. The pellet was redissolved in M/15 phosphate buffer at pH 7.0 and the solution run again in the ultracentrifuge under the same conditions. The resulting pellet was resuspended in about 0.1 ml of buffer and was fed to previously starved *M. persicae* through a plastic membrane. None of these aphids became infectious.

An attempt was then made to isolate the virus from the insect vectors. Almost 3 g (estimated at 10,000) of *M. persicae* that had been feeding on infected potatoes for 2 weeks were washed from the plants. These aphids were ground with 5 ml of 0.05 per cent. gelatine in distilled water chilled in ice. The macerated insects were then centrifuged at 10,000 *g* for 10 min in a refrigerated centrifuge. The supernatant was centrifuged at 40,000 *g* for 30 min in a Spinco ultracentrifuge. The pellet was designated fraction 1. The supernatant was then centrifuged for 30 min at 100,000 *g* and the pellet designated fraction 2. The greater part of both pellets was easily resuspended in 0.05 per cent. gelatine. Solutions from fractions 1 and 2 were hand-inoculated on to *P. floridana* leaves, fed to aphids through plastic membranes, and inoculated into the haemocoel of normal aphids. Infections were obtained only from inoculation of fraction 2 into aphids and then in only 2 of 18 plants on which the inoculated aphids were permitted to feed.

This experiment was repeated using aphids from infected *P. floridana*. The supernatant from the preliminary low speed centrifugation at 10,000 *g* for 30 min was followed by ultracentrifugation at 100,000 *g* for 30 min and the pellet resuspended in 0.05 ml of 0.05 per cent. gelatine. This was injected into aphids of which 30 survived. When these were placed singly on *P. floridana* indicator seedlings for 3 days, three infections were obtained. The virus can therefore be recovered from infected aphids.

It is suggested that the apparent inability of aphids to acquire leaf roll from solution is due to the fact that too little active virus was present in the solution for it to infect the aphid vectors.

(vi) *Possibility of Multiplication of Potato Leaf Roll Virus in the Aphid.*—All of the experiments reported in the previous pages are explicable on the hypothesis that an aphid reinjects into a plant only a fraction of the virus it ingests and that no multiplication of virus need occur in the insect. Maramorosch (1953) has concluded that leafhopper-borne viruses that have a latent period undergo multiplication in their vectors. It is therefore important to determine whether multiplication of viruses can also occur in aphids. Unfor-

TABLE 4

TRANSMISSION OF POTATO LEAF ROLL VIRUS BY A CLONE OF *M. PERSICAE*, INOCULATION FEED ON A SINGLE INFECTED PLANT OF *D. TATULA*

Duration of Acquisition Feed (hr)	Period on Chinese Cabbage (hr)	Duration of Inoculation Feed (hr)	Infections	
			Number	Percentage of Seedlings Inoculated
1	24	4	0	0
1	48	4	0	0
1	72	4	0	0
1	96	4	1	3
3	0	4	1	3
3	24	4	3	5
3	48	4	6	9
3	72	4	15	21
3	96	4	12	17
3	144	4	14	20
6	24	4	3	10
6	48	4	10	28
6	72	4	10	28
6	96	4	15	42

tunately many of the techniques found satisfactory for demonstrating multiplication of viruses in leafhoppers are inapplicable to aphids. For example, leaf roll virus is not transmitted by a viviparous aphid to its offspring. This was reported by Elze (1927) and Smith (1929) and has been repeatedly confirmed with the local virus and vector. Inoculation of the aphid (see above), though occasionally successful, was too uncertain to permit the technique, so successfully applied by Maramorosch (1952), to be used with aphids. Heat treatment used by Kunkel (1937) to render infective leafhoppers free from virus could not be employed with *M. persicae* because of the proximity of the thermal death points

of the aphids (Broadbent and Hollings 1951) and that of the virus (Kassanis 1950). With so short a latent period it was not easy to devise an experiment to measure the relation of the dosage of the virus to the duration of the latent period; and techniques for measuring virus concentration were too inaccurate to permit measurement of an increase in virus concentrations in the insect during the latent period.

In view of the difficulties outlined in the previous paragraph, the following experiment was designed in an attempt to obtain data on the possibility of the multiplication of potato leaf roll virus in *M. persicae*. Large numbers of aphids were given short acquisition feeds (1, 3, and 6 hr) on leaf roll infected *Datura tatula*. They were then removed to Chinese cabbage which is immune to potato leaf roll. At 24 hr intervals groups of aphids were placed singly on *P. floridana* indicator seedlings in the two-leaf stage and the percentage of infective aphids determined (Table 4).

Precautions were taken to ensure that aphids selected at the later stages were not progeny of those placed on the Chinese cabbage. One transmission following a latent period of 7 hr was obtained. The percentage of transmission continued to increase for 3 days, in spite of the fact that the source of virus was removed after 3 hr. The most likely explanation is that the concentration of virus in the haemolymph continued to increase after the ingested virus was distributed through the tissues of the vector.

(c) The Occurrence of a Latent Period

The data in the previous section confirm the view that the virus of potato leaf roll is transmitted by the "biological" mechanism, and suggest that limited multiplication of the virus may occur in the aphid. If multiplication does occur a latent period would be expected, and studies were therefore undertaken to attempt to resolve the conflicting reports on the existence of a latent period.

(i) An experiment duplicating in all published details that outlined in Table 7 of Kirkpatrick and Ross (1952) resulted in no transmission, although these authors reported transmission by 4 out of 5 aphid colonies.

A similar experiment (except that the 12 aphids were moved every 1½ hr during the day and were placed at night on individual Chinese cabbage seedlings) was carried on for 4 days, and it was only on the last day that infections were obtained. Many other results confirm the conclusion that "mechanical" transmission does not occur. Day and Irzykiewicz (1954) have demonstrated that mechanical transmission by aphids is most efficient when the acquisition and inoculation feeding periods approximate 2 min each. Hundreds of tests using 5 min feeding periods have not resulted in a single inoculation of potato leaf roll virus.

(ii) Larson (personal communication) found with both "mild" and "severe" strains of leaf roll about 20 per cent. transmission with acquisition feeds and inoculation feeds of 60 min each. But when an experiment using the same conditions was performed, no infections occurred with our strain of *M. persicae* in the short feeding periods (Table 5).

It appeared from the above results that under most conditions a latent period actually occurs in the transmission of potato leaf roll virus. Two experiments were performed to determine the duration of this latent period. The results are presented in Table 6. It would seem that the latent period under these conditions approximates 20 hr at 25°C. However, it was nearer 3 days in the experiment described in the preceding section and only a few hours in Table 4. The results confirm conclusions of Kassanis (1952) and of MacCarthy (1954) that the latent period is very variable. The explanation of this variability will be put forward in Section IV.

The above experiments dispose of two of the possible explanations advanced in Section I. The use of *P. floridana* and *D. tatula* as source plants did not generally result in transmissions except following a long latent period. Similarly, transmission in these experiments never occurred first by a mechanical

TABLE 5

TRANSMISSION OF POTATO LEAF ROLL BY *M. PERSICAE* FROM *D. TATULA* TO *P. FLORIDANA* (THREE-LEAF STAGE) AT 22°C. 10 APHIDS PER TEST PLANT

Duration of		Number of Infections
Acquisition Feed	Inoculation Feed	
10 min	10 min	0/36*
60 min	60 min	0/36
120 min	120 min	0/72
24 hr	24 hr	17/22

* Denominator indicates the number colonized, the numerator the number that became infected.

and later by a biological mechanism, and this suggestion certainly does not explain the conflicting results on the occurrence of the latent period. Two other possibilities were put forward, namely that the results were explicable by differences in the viruses used, or by differences in the vectors. These possibilities will now be considered.

(iii) *Vector Relationships of Strains of Leaf Roll Virus*.—The occurrence of “strains” of leaf roll has been clearly established by Webb, Larson, and Walker (1952). It is possible that certain strains may have vector relationships differing from the typical strain. Some evidence that Klostermeyer (1953) was dealing with a virus differing from typical leaf roll is suggested by his statement that “visitors from several eastern States and from foreign countries declare such pronounced symptom expression does not occur elsewhere.”

In order to test the possibility that more than one virus causing leaf rolling symptoms was present in Australia, leaf roll infected potatoes were obtained from widely separated localities and each of these was tested in the manner

of the experiment set out in Table 5. Of 41 isolates examined none reacted differently from the typical strain. Details of the isolates examined are as follows:

- 2 isolates from A.C.T. from varieties Sebago and Katahdin,
- 6 isolates from four districts in New South Wales from varieties Exton, Factor, Katahdin, Saranac, and Sebago,
- 7 isolates from six districts in Victoria from varieties Sebago and Sequoia,
- 16 isolates from three localities in South Australia in varieties Adina, Delaware, Exton, Katahdin, Kennebec, Monak, and Sebago, and
- 10 isolates from five localities in Tasmania in varieties Woolnorth and Medium Brownell.

TABLE 6

LATENT PERIOD OF POTATO LEAF ROLL VIRUS IN *M. PERSICAE* AT 27-28°C. ACQUISITION FEED ON INFECTED *P. FLORIDANA*. INOCULATION FEEDS ON *P. FLORIDANA* IN TWO-LEAF STAGE

Acquisition Feed (hr)	Inoculation Feed (hr)	Total Time (hr)	Infections (out of 36)
15	4	19	0
22	4	26	3
28	4	32	1
39	4	43	3
51	4	55	7
15	3	18	0
15	5	20	2
15	7	22	8
15	9	24	0
15	11	26	2
15	13	28	6
15	24	39	10
15	29	44	10

It is, of course, virtually impossible to prove that only one virus causing the symptoms of potato leaf roll occurs, but the suggestion that two such viruses account for the divergent results published on the latent period of the virus in the vector is made less attractive by the fact that Larson (personal communication, see above) has found that transmission of two of his strains was practically the same when they were compared by the method set out in Table 5. Certainly no evidence has been obtained in the present work of the existence of two leaf rolling viruses with different vector relationships.

(iv) *Differences in Strains of Vectors*.—Although most of the results presented so far in this paper confirm the views of Elze, Smith, and Kassanis, a few transmissions were obtained following short acquisition and inoculation feeds. One such instance is shown in the 3-hr acquisition feeds of Table 4.

But such instances of latent periods of only a few hours were very infrequent by comparison with those reported by the American workers.

These and other results suggested that differences in strains of aphids might account for the reported differences in duration of the latent period. All work so far described in this paper was done with aphids from a colony of *M.*

TABLE 7

TRANSMISSION OF POTATO LEAF ROLL VIRUS BY CLONES OF *M. PERSICAE* IN FOUR SEPARATE EXPERIMENTS

Clone	Ability to Transmit			
	Number Infected (out of 4) in 24-Hr Test	Number Infected (out of 36) in:		
		5-Day Test	4-Day Test	6-Day Test
1	0	1	9	16
2	0	1	6	18
3	0	2		
4	0	2		
5	0	4		
6	0	10		
7	0	15		
8	0	17		
9	1	4		
10	1	8		
11	1	12		
12	3	0	6	14
13	3	4	12	23
14	4	1		
15	4	3		
16	4	4		
17	4	5		
18	4	14		
19	4	16	3	23
20	4	17	5	25

persicae that had been maintained in the laboratory for several years. A new colony was initiated from a number of field-caught alates, and clones were selected from this colony by the following method. Mature apterae were permitted an acquisition feeding period on a leaf roll infected *P. floridana* plant for 3 to 5 days. They were then placed singly for 24 hr on *P. floridana* indicator seedlings and moved daily to new seedlings for 4 days. The results of 205 aphids were as follows: 35 transmitted to all of the four indicators, 68 to three indicators, 58 to two indicators, 31 to one indicator, and 13 failed to transmit to any of the four indicator seedlings. Each surviving aphid was then placed

on a Chinese cabbage seedling to propagate the clone. Twenty of these clones were then studied further in the following way. Approximately 50 aphids from each clone were placed for 3 days on uniform, infected *P. floridana* plants; they were then placed singly on 36 *P. floridana* indicator seedlings for 2 days and the percentage of infectious aphids determined. The results (Table 7) demonstrate that selection based on one test did not influence the performance of the clones in a subsequent test. This suggests that the major component in apparent differences in vector efficiency is the operation of chance differences presumably in feeding, but the magnitude of the differences between the performance of, for example, clones 1 and 8 and between clones 14 and 20 suggested that real differences in vector efficiency may exist between clones. However, subsequent tests with these selected clones failed to substantiate this suggestion and it is apparent from columns 4 and 5 of Table 7 that the preliminary selection of clones did not result in strains of aphids that differed in vector ability. A latent period experiment similar to that set out in the lower part of Table 6 was carried out with clones 1 and 19. No differences were found.

It has thus not proved possible to isolate from the material available clones of *M. persicae* that differ in their ability to transmit potato leaf roll virus. However, the experiments demonstrate the marked variability in transmission which can occur within an experiment and that large numbers of vectors must be tested before conclusions are justified concerning the efficiency of clones to transmit the virus.

Simons (1954) reported considerable differences in the ability to transmit pea enation mosaic virus between young and mature apterae. Most workers have used mature apterae, but it seemed possible that differences in the stages used may have accounted for some of the differences in the results reported by various authors. However, a test with young and mature apterae transmitting potato leaf roll virus showed that both groups transmitted with equal efficiency. In a comparative experiment the mature apterae transmitted the disease to 18 of 72 indicators, and the young apterae to 16 of 72 indicators.

The suggestion that strains of aphids differing in their vector efficiency may explain the differences in the latent period reported by European and American workers cannot be studied by using aphids available to us. It remains, however, the most attractive hypothesis.

IV. DISCUSSION

Insects transmit viruses by several mechanisms (Day 1955); "direct mechanical transmission" is effected by mosquitoes transmitting rabbit myxomatosis; "modified mechanical transmission" is effected by aphids transmitting mosaic viruses; "delayed mechanical transmission" has been suggested as a designation for those viruses transmitted by ingestion and subsequent excretion by the vector; finally, "propagative transmission" is the type of transmission in which the virus is ingested, multiplies in the vector, and the "offspring" of the infecting virus are released. The first two types include the "vector-direct" viruses

as defined by Day and Irzykiewicz (1954). Potato leaf roll virus is clearly excluded from this category for the following reasons:

- (i) It is transmitted by an aphid following a moult.
- (ii) It can be isolated from the haemolymph.
- (iii) The vector is infective for many days.
- (iv) Vector specificity is well marked.
- (v) There is often a latent period between an acquisition feed before a vector is capable of transmitting the virus.

Potato leaf roll is, thus, not a vector-direct virus, as defined by Day and Irzykiewicz (1954). Tables 4 and 6 provide data relevant to a decision between the delayed mechanical and the propagative types of transmission:

- (1) The ability of the vector to transmit would be proportional to the duration of the acquisition feed in delayed mechanical transmission but would be unrelated in propagative transmission. Results with potato leaf roll virus (Table 6) favour the second alternative.
- (2) The maximum efficiency of transmission should occur with shorter intervals between acquisition and transmission with delayed mechanical transmission but should increase with time in propagative transmission. The data of Tables 4 and 6 again confirm the second alternative.
- (3) The frequency distribution of the number of infections plotted against the duration of the inoculation feeding period would follow a normal distribution curve if transmission was by the delayed mechanical mechanism, but would follow an exponential curve with propagative transmission. In this also the data of Tables 4 and 6 confirm the second alternative.

These considerations taken together indicate strongly that the potato leaf roll virus multiplies to a limited extent in the aphid vector. The route of the virus in the vector has been traced as far as the haemolymph, but not into the salivary glands or saliva. Blattny (1931) reported changes in the cytology of the aphid salivary glands after infection with leaf roll, but efforts to confirm this report have been unsuccessful.

The differences reported in the literature on the occurrence of a latent period are explained mainly by differences in the vectors used, in conjunction with the efficient source plants *P. floridana* and *D. tatula* used by recent investigators. The latent period is thus the time required for the virus to move from the midgut to the saliva of the vector. The data suggest the hypothesis that the virus decreases in activity as it moves along this path. When the amount of virus ingested is small, sufficient to reach the saliva is not present until multiplication has occurred. When the amount ingested is large some of it may occasionally reach the saliva before multiplication has occurred. But the barriers between midgut and saliva may differ in effectiveness between strains of aphids and, even with sources of relatively high virus concentration, sufficient to reach the saliva may not be ingested in those strains in which a latent period is demonstrable. Watson (1940) is correct in concluding that no fixed latent period occurs in several aphid-borne persistent viruses, and it

is now suggested that this may be due to the strains of the vector as well as to the characteristics of the virus.

It appears from published data that the mechanism of transmission of certain other viruses, e.g. beet yellows (Watson 1940), beet yellow-net (Sylvester 1949), and carrot motley dwarf (Stubbs 1948) may be similar to that of potato leaf roll. There may, in fact, be a series of aphid-borne viruses between these and such viruses as that of the lily symptomless disease described by Brierley and Smith (1944) and even that of strawberry virus 3 (Prentice and Woolcombe 1951), which has a very long latent period in its vector.

Leafhopper-borne viruses, with the exception of that causing beet curly-top, have longer latent periods in their vectors and multiplication of virus in the vector has been demonstrated in a number of them (Black 1953). On the basis of published work on the transmission of beet curly-top by *Circulifer tenellus* (Baker) it seems likely that the mechanism of transmission in this vector is similar to that described for potato leaf roll.

It will be apparent from the above that the occurrence of a very short latent period is not incompatible with the view that a virus is transmitted by the vector-latent mechanism, or even with multiplication of the virus in the insect vector. Black (1950) suggested the generalization that "most, if not all, plant viruses with *long* incubation (= latent) periods in their leafhopper vectors multiply in those vectors." Further work with beet yellows, beet yellow-net, and carrot motley dwarf virus may well permit the extension of this generalization to state that plant viruses with latent periods in their vectors multiply in those vectors.

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FUNCTIONAL DIFFERENTIATION OF THE HINDGUT EPITHELIUM OF THE BLOWFLY LARVA INTO LONGITUDINAL BANDS

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Summary

The hindgut of blowfly larvae commences a short distance posterior to the entry of the malpighian tubules into the alimentary canal. It is divided into short anterior and posterior regions, which function principally as sphincters, and a long central region. The central hindgut is composed of three histologically distinguishable cell types, each forming a longitudinal band. The bands produced by cell types *A* and *B* each form about one-half of the circumference of the hindgut, whereas the cells of type *C* form two narrow longitudinal strips, each one cell wide, situated at the junctions of the bands of *A* and *B* cells.

The *A* cells are rich in potassium, acid phosphatase, dehydrogenases, and acetyl esterase. On the other hand, the *B* cells react strongly for ammonia and stain diffusely for barium in larvae fed on a barium-enriched diet. The *C* cells react very strongly to the test for ammonia and accumulate barium-rich granules.

The hindgut epithelium rapidly takes up ammonia directly from the haemolymph and the *B* and *C* cells are particularly active in this process. Much of the ammonia appears to be eliminated as bicarbonate.

Evidence is steadily accumulating that the hindgut epithelium is an active tissue which plays an important role both in metabolism and in regulating the products of excretion.

I. INTRODUCTION

The hindgut of insects is no longer regarded merely as a passage for the discharge of food residues from the midgut to the exterior. Except for sphincters at the anterior and posterior ends it appears, in a few species, to be of uniform structure throughout. In the majority of species, however, there is some degree of morphological differentiation, which most commonly takes the form of differences in diameter of various regions. Specialized rectal pads are also present in many insects. These play an important part in the process of absorbing water from the food residues before discharge and may also be concerned in recovering other valuable constituents (Wigglesworth 1953). Ramsay (1950) has shown in *Aedes detritus* that the anterior part of the rectum has a different epithelium from the remainder (a distinction not found in *A. aegypti*) and that this is correlated with the ability of the former but not the latter species to produce a hypertonic fluid in the rectum. A few papers also describe restricted distributions in the insect hindgut for alkaline phosphatase, iron, strontium, and barium (Waterhouse and Day 1953).

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Following the demonstration of a striking functional differentiation of various groups of cells in the midgut of blowfly larvae (Waterhouse and Stay 1955), similar techniques were applied to the larval hindgut about which almost nothing was known. It is shown here that, although the hindgut is not as involved as the midgut in absorption and intermediary metabolism, nevertheless, it is a very active organ. Furthermore, for the greater part of its length it is highly differentiated both histologically and functionally into longitudinal bands, each formed from one of three distinct cell types.

II. METHODS

Almost mature third instar larvae of the Australian sheep blowfly *Lucilia cuprina* (Wied.) reared at 30°C on a standard artificial medium (Waterhouse and Stay 1955) or on liver were used in all experiments.

Histological observations were made on material fixed in alcoholic Bouin's fluid and stained with either Mallory's triple connective-tissue stain, Delafield's haematoxylin and eosin, or Bodian's protargol silver method. Regaud's method was used for staining mitochondria.

Histochemical tests for the following were performed in a manner already described (Waterhouse 1951; Waterhouse and Stay 1955): lipoid, glycogen, iron, copper, barium, and strontium, potassium, ascorbic acid, dehydrogenases, and cytochrome oxidase. Tests were also performed for zinc (dithizone—Mager, McNarry, and Lionetti 1953), uric acid (methenamine-silver nitrate—Gomori 1952), amine oxidase (Blaschko and Hellmann 1953), phenol sulphatase (Rutenburg, Cohen, and Seligman 1952), and lipase (the "Tween" 20, 60, and 80 substrates of Gomori (1952) after fixation in cold formalin). In addition, the following tests were carried out:

(a) *Ammonia*

Larvae were dissected rapidly under saline and then immersed either in Nessler's or Riegler's reagents (Lennox 1941a; Waterhouse 1950). The characteristic orange-brown reaction product of Nessler's reagent with ammonia is sufficiently stable when tissues are passed rapidly through the higher alcohols and xylene for its distribution to be examined in cleared whole mounts or after paraffin sectioning. Fading occurred fairly rapidly in xylene, tetrachloroethylene, and balsam prepared with these solvents, but there was sufficient time to obtain a photographic record if desired.

Other tissues were transferred after several minutes in Riegler's reagent to saturated aqueous calcium hydroxide, whereupon regions containing ammonia turned red. Riegler's reagent, as in earlier tests, appeared to be considerably less specific than Nessler's reagent.

(b) *Carbonate or Bicarbonate*

The evolution of carbon dioxide on addition of 5N sulphuric acid was detected by absorbing it, in the apparatus described by Feigl (1947), in a dilute (about 0.005N) sodium carbonate solution coloured red with phenol-

phthalein. The formation of bicarbonate was indicated by decolorization of the indicator. After the macerated tissue and acid had been introduced into the apparatus they were mixed by means of a magnet and stirring bead. Gas evolution was assisted by gentle warming in a light beam.

(c) *Phosphatases*

In addition to the Gomori techniques (Gomori 1952) using sodium glycerophosphate, the α -naphthyl phosphate-azo dye methods of Gomori (1952) for alkaline phosphatase and of Burton (1954) for acid phosphatase were employed. Initial fixation for 2-6 hr in ice-cold, buffered, 10 per cent. formaline preserved the tissues better than alcohol or acetone, without otherwise influencing the results. However, even with formalin fixation, considerable disintegration occurred in the ammonium sulphide step of the glycerophosphate method. Brief (5-10 min) fixation in alcoholic Bouin immediately before this step prevented disintegration, apparently without producing any artefacts.

(d) *Acetyl Esterase*

The α - and β -naphthyl acetate techniques for aliesterase activity, using either Diazo Fast Blue RR or Diazo Blue B were employed (Gomori 1952; Ravin, Zacks, and Seligman 1953). The substrate-dye mixtures were chilled with ice during incubation and replaced every 15-20 min for 1-2 hr. Tissues were fixed in neutral buffered formalin and either mounted in glycerogel or, alternatively frozen sections were prepared from tissues embedded in gelatin.

III. RESULTS

(a) *Morphology and Histology of the Hindgut*

In the *L. cuprina* larva the hindgut and the midgut (relative lengths 2 : 3) are together some five to six times the length of the body, a figure which is unusually high compared with that for most other insects. This may be related to the high efficiency demanded of the blowfly alimentary canal during the very short larval growth period.

Although it is a convenient, broad generalization in insects to regard the hindgut as commencing at the point of discharge of the malpighian tubules into the digestive tract, this is not always so. In *L. cuprina* larvae, sections show that midgut cells extend for a short distance posterior to this point (Fig. 1, X). The hindgut first narrows for a short sphincter region and then expands slightly to maintain a more or less constant diameter (varying slightly with distension by food) until it terminates with a narrower sphincter region at the anus. It first pursues a winding course forwards from near the posterior end of the larva to the vicinity of the proventriculus and then backwards to the anus, receiving its tracheation from the same main trunks which supply the adjoining loops of the midgut. Numerous intracellular tracheae and tracheoles, which run both circularly and longitudinally through the epithelium, are a constant feature of the hindgut and are very clearly seen in Bodian preparations (Plate

1, Fig. 2). The peritrophic membrane extends continuously throughout the hindgut and is discharged undamaged at the anus, extruded lengths being detached mechanically from time to time (Waterhouse 1954). The pH of the hindgut contents is uniform throughout and lies in the range 7.8-8.0 (Waterhouse 1940).

The characteristic histology of the three regions of the hindgut epithelium is indicated in the inserts in Figure 1.

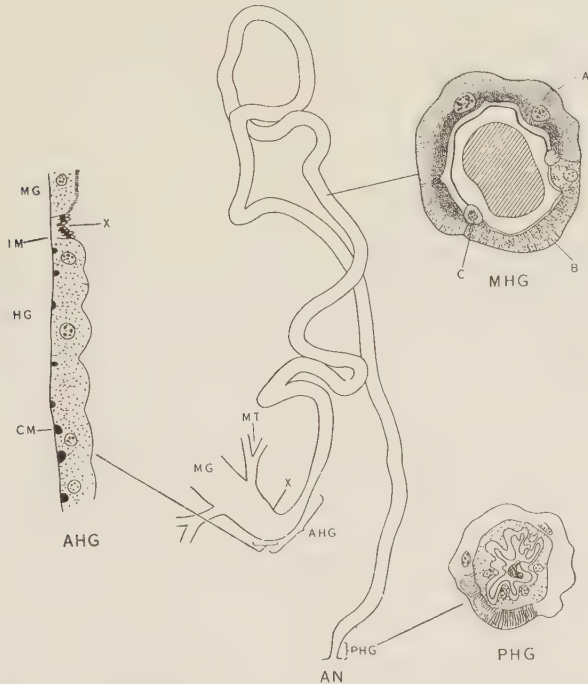


Fig. 1.—Diagram of the hindgut of a *L. cuprina* larva with inserts indicating the typical histology of the various regions. AHG, anterior hindgut (longitudinal section); MHG, central hindgut; PHG, posterior hindgut; A, B, C, cell types in central hindgut; CM, circular muscles; HG, hindgut; IM, imaginal cells; MG, midgut; MT, malpighian tubules; X, junction of mid and hindgut.

(i) *Anterior Hindgut (AHG)*.—The hindgut commences where the alimentary canal diminishes sharply in diameter (Fig. 1, X); it is separated from the midgut by a ring of small imaginal cells which later form portion of the adult hindgut. The first true hindgut cells contain many intracellular tracheoles (Plate 1, Fig. 2) in contrast with the midgut cells where these are comparatively rare. The hindgut cells in this region all have a similar appearance, the nucleus is basal or median, the cytoplasm is uniform, and there is rather narrow cuticular lining. With Mallory's stain the basal cytoplasm is somewhat fuchsino-philic, the apical half of the cell staining predominantly with aniline blue. The

first short zone of the hindgut is richly supplied with muscles, particularly with circular muscles. These form both a sphincter and a means for marked peristaltic activity when food residue is passed on from the midgut. The circular muscles are invaginated into the base of the epithelial cells at the rate of two or three groups of muscles per cell (Fig. 1, *CM*; Plate 1, Fig. 2). This arrangement gives the impression, in $10\ \mu$ transverse sections, that the muscles are intracellular.

(ii) *Central Hindgut*.—The histology of the hindgut is apparently uniform from a short distance posterior to the commencement of the hindgut proper to the anal sphincter. Throughout this region the epithelium is differentiated into three cell types (Table 1; Fig. 1, *MHG*). These types can also be distinguished in the freshly-hatched larva.

Cell type *A*.—These cells usually form rather more than half of the circumference of the hindgut, although they may constitute as little as one-third (Plate 1, Fig. 1). They have an apical nucleus, are typically higher, and possess a wider cuticular lining than the two other cell types present. The apical cytoplasm contains both basophilic and fuchsinophilic material and is granular, whereas the basal cytoplasm is vertically striated and stains relatively lightly, except with Bodian. Numerous rod-like and filamentous mitochondria are present in the basal cytoplasm, whereas they are scarce in the apical portion of the cell.

Cell type *B*.—These cells generally occupy less than half (sometimes up to two-thirds) of the hindgut circumference (Plate 1, Fig. 1). They have a basal or median nucleus and a relatively narrow cuticular lining. The cytoplasm is finely granular and is not differentiated into apical and basal zones by the staining methods employed. Mitochondria are fairly abundant and are distributed evenly throughout the cytoplasm.

Cell type *C*.—These cells, which are much smaller than the other types, are situated at the junction of the bands of *A* and *B* cells (Plate 1, Fig. 3), and form two narrow longitudinal strips each one cell wide. Each *C* cell is longitudinally elongated, tapers at both ends, and has a length equivalent to that of several *A* or *B* cells (Plate 1, Fig. 6). Type *C* cells do not always touch each other, so that the two strips of *C* cells are not represented on either side of all sections. Occasional mitochondria are distributed throughout the cytoplasm.

(iii) *Posterior Hindgut*.—Shortly before its termination at the anus the epithelium becomes uniform again (Fig. 1, *PHG*). The cuticular lining becomes relatively thick and the circular musculature is well developed to form an anal sphincter.

No spines have been observed in the cuticular lining of any region of the larval hindgut. These are a feature of the adult hindgut in many higher Diptera.

(b) *Histochemical Tests*

(i) *Lipoid*.—No osmium tetroxide-reducing materials were detected in the hindgut epithelium, irrespective of the larval diet. After formalin fixation and Sudan black B treatment the hindgut cells were lightly and uniformly stained,

this being due to a general ground coloration of the cytoplasm. When such preparations were slit longitudinally and mounted flat, the *C* cells appeared to be slightly more heavily stained than the *A* or *B* cells. With Nile blue, a rather generalized, non-specific staining resulted. Thus, there was no indication that deposits of lipid were accumulated by the hindgut epithelium.

TABLE 1

COMPARISON OF THE THREE CELL TYPES FORMING MOST OF THE HINDGUT OF *L. CUPRINA*

Cell Part and Staining Reactions	Cell Type		
	<i>A</i>	<i>B</i>	<i>C</i>
Proportion of circumference	$\frac{2}{3}$ to $\frac{1}{2}$	$\frac{1}{3}$ to $\frac{1}{2}$	Nil to very small
Height	Relatively high	Generally lower than <i>A</i>	Generally $\frac{1}{3}$ to $\frac{1}{4}$ of <i>A</i>
Nucleus	Apical	Basal or median	Apical, smaller than <i>A</i> or <i>B</i>
Cuticular lining	Wide	Narrow	Very narrow
Cytoplasm:			
Apical	Finely granular	Weak vertical striations	Finely granular
Basal	Vertical strong striations	Finely granular	Finely granular
Mitochondria	Mainly basal	Evenly distributed	Sparse, even
Mallory:			
Apical	Fuchsinophilic	} Uniformly stained with all components	} Weakly fuchsinophilic
Basal	Aniline blue predominates		
Bodian:			
Apical	Lightly stained	} Uniform light, with few scattered black granules	} Uniform light
Basal	Sometimes more heavily stained; median zone of black granules at times		
Haematoxylin-eosin:			
Apical	Basophilic; cell processes extend into cuticular lining	} Staining intermediate between the two regions of <i>A</i>	} As for <i>B</i> or slightly more basophilic
Basal	Light staining		

(ii) *Glycogen*.—After freeze-drying, the cytoplasm of all cell types stained a little more deeply with the periodic acid—Schiff (PAS) test before than after digestion by saliva, although no difference was observed after ordinary fixation. PAS-positive granules were seldom seen and it is evident that little or no glycogen is stored in the hindgut epithelium. The circular muscles forming

the sphincter at the anterior end of the hindgut were found to contain some glycogen.

Staining, which proved resistant to digestion by saliva, occurred in the cuticular lining and the basement membrane of the hindgut epithelium and also in the peritrophic membrane. This staining was virtually abolished by acetylation (McManus and Cason 1950), indicating that these structures contain carbohydrate other than glycogen.

(iii) *Potassium*.—When fresh hindguts were tested for potassium one side of the middle region stained much more heavily than the other. The anterior and posterior regions only stained lightly. The deeply staining side of the middle region consisted of A cells. The basal, vertically-striated cytoplasm of these cells stained heavily although the apical cytoplasm also gave a positive reaction (Plate 1, Fig. 5). Numerous large granules, some black or brown, some yellow, occurred in the A cells, being concentrated in the median zone of the cell. The B cells stained very lightly and contained relatively few, scattered granules similar to those in the A cells. These granules tended to be concentrated at the base of the B cells. The C cells reacted very weakly, staining about as intensely as the apical cytoplasm of the A cells or failing to stain.

The addition of 2.5 mg KCl per g medium slightly intensified the staining reaction without altering its character or distribution.

(iv) *Barium and Strontium*.—Neither barium nor strontium could be detected in the hindgut epithelium of larvae fed on the standard medium.

However, after the addition of 0.25 mg BaCl₂ per g medium, but not after the addition of a similar amount of SrCl₂, one side of hindgut epithelium often stained pink with rhodizonate. Furthermore, portions here and there of the bands of C cells stained very heavily (Plate 1, Fig. 4).

Sections demonstrated that the cytoplasm of the B cells was staining uniformly and lightly for barium, but that the A cells were not reacting. The C cells at times contained many small, dark red granules (Plate 1, Fig. 6) and sometimes also clusters of large, brownish red, rod-shaped crystals concentrated near the gut lumen. At other times the C cells stained lightly or failed to react.

A characteristic feature of larvae fed on barium-enriched food was the presence of large, light brown crystals attached to the hindgut epithelium and protruding into the gut lumen. These crystals, which were often but not always associated with the C cells, generally failed to react with rhodizonate.

(v) *Ammonia*.—Nessler's reagent rapidly produced a conspicuous red-brown coloration for ammonia along most of the central hindgut of feeding larvae, whereas the anterior and posterior hindguts were unstained (Fig. 2(a)). In the central hindgut a striking reaction often occurred along one side only, whereas the other side was unstained or comparatively lightly stained. The intensity of staining increased progressively along the first half of the central hindgut to remain at a fairly uniform high level throughout the second half.

When the staining was weaker than usual it appeared to be largely restricted to a narrow, not necessarily continuous, band on either side of the central hindgut. On the other hand, when the staining was particularly intense, the reddish brown precipitate occurred not only throughout the entire epithe-

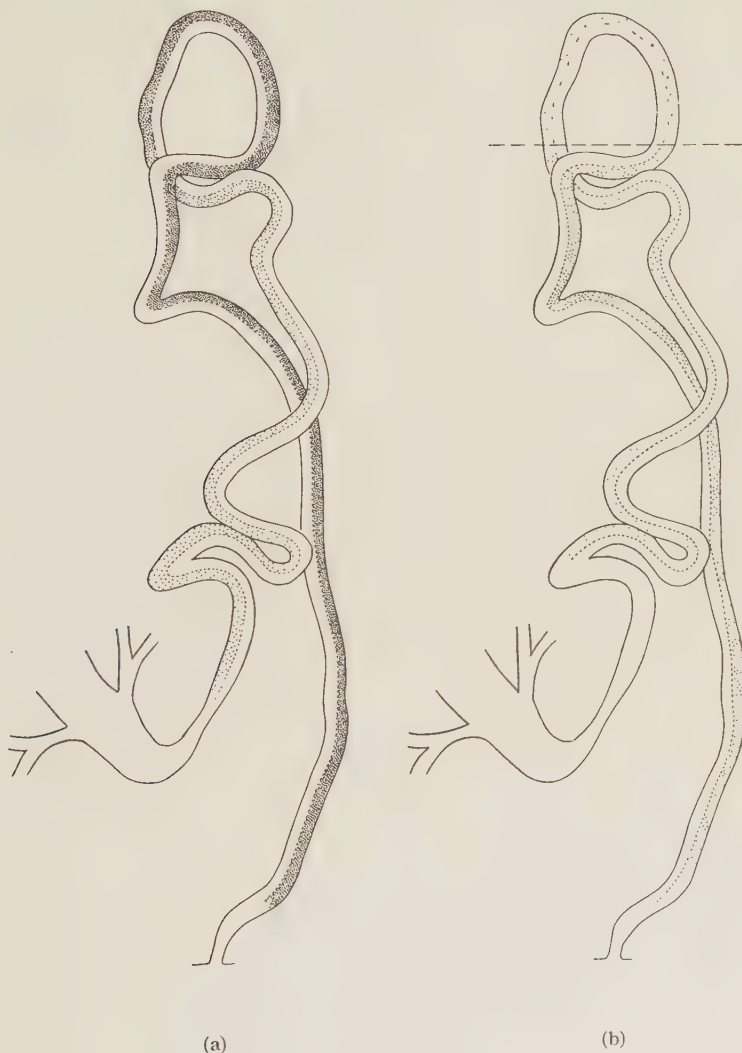


Fig. 2.—The distribution of ammonia (intensity indicated by stippling) in the hindgut of *L. cuprina*. (a) Actively feeding larva; (b) ligatured larva, injected posterior to dotted line (see text).

lium, but also on the outside of the gut. This suggests that there is a tendency for ammonia to diffuse out of the gut during manipulation. Lennox (1941a) recorded changes in intensity of staining along the hindgut but not any tendency for the main staining to occur along one side.

Riegler's reagent at first produced a deeper stain along one side of the central hindgut than along the other, but this distribution was soon lost with the development of a more generalized, apparently non-specific staining throughout the larva.

In typical sections of Nessler-stained hindgut there was an intensely coloured band formed on either side of the gut by the *C* cells, together with the immediately adjoining portions of the bands of *A* and *B* cells (Plate 2, Fig. 1). The cytoplasm of this region was yellowish brown and contained many small, dark brown granules. Elsewhere the cytoplasm of the *A* cells was colourless, although large brown granules sometimes occurred in the cuticular lining. By contrast, in the *B* cells a positive reaction almost always occurred which involved all apical and basal structures. The *C* cells generally stained heavily to very heavily, although at times they gave a very weak or negative reaction. Nuclei of all cells usually failed to react.

In sections of very heavily stained hindguts the entire cytoplasm of the *B* cells was brown and contained numerous dark brown granules (Plate 2, Fig. 2). The *A* cells also stained similarly, but usually less intensely.

These results raised the question of the route of ammonia excretion from the body, i.e. whether the hindgut ammonia came either via the malpighian tubules, as claimed by Lennox (1941*a*), or by direct uptake by the hindgut from the haemolymph as suggested by Hurst (1941). When larvae were tested at varying intervals after transfer from their food to individual containers with 1.5 per cent. agar, the ammonia reaction diminished progressively as their guts emptied of food. Under these conditions the areas in the vicinity of the *C* cells retained their strong positive reaction for longer than elsewhere. After several hours the hindgut generally reacted only weakly.

When larvae in the latter condition were ligatured so that a short loop of the hindgut was excluded from the posterior portion (Fig. 2(*b*)) and either blood from actively feeding larvae or 1 per cent. ammonium chloride in saline injected into the posterior portion, the strong positive reaction was re-established after a few minutes in the posterior portion only. This is clear evidence that ammonia is taken up directly from the haemolymph, at least by the posterior segment of the central hindgut.

No ammonia was detected in the malpighian tubules in this test, or for that matter with Nessler's reagent in actively feeding larvae, although it is known from other tests to be present in the tubules (Lennox 1941*a*; Waterhouse 1950). It is, however, not possible to say whether the excretion from the malpighian tubules carries with it any important quantity of ammonia.

(vi) *Carbonate or Bicarbonate*.—When a dissected alimentary canal was flooded with dilute sulphuric acid and pricked at intervals along its length with a fine pin, bubbles of gas were produced from the central hindgut and particularly from the posterior half of this region. The hindguts of larvae from batches which gave a particularly intense reaction for ammonia also produced many bubbles of gas, whereas those from batches giving a weak Nessler reaction evolved relatively little gas. Medium on which larvae had fed for several

days, and also the crop contents of these larvae, gave a strong Nessler reaction and evolved gas on acid treatment. Elsewhere the alimentary canal produced no gas.

The sodium carbonate-phenolphthalein test demonstrated that the gas evolved was carbon dioxide. Cyanides, azides, sulphides, sulphites, and thio-sulphates were prevented from interfering by the methods of Feigl (1947). Control tests using all larval tissues except crop, malpighian tubules, and hindgut indicated that little or no carbon dioxide was evolved under these conditions. Brown (1938) recorded the presence of ammonium bicarbonate in the food on which larvae had grown for several days and it can probably be assumed that the same compound is present in the larval body.

(vii) *Phosphatases*.—Alkaline phosphatase activity was very weak or absent, regardless of whether the entire hindgut or sections of it were incubated, and of whether the substrate used was glycerophosphate or β -naphthyl phosphate. On those occasions when very weak activity was observed, staining appeared to be a little more distinct in the *C* than in the *A* or *B* cells. The peritrophic membrane and its contents sometimes stained fairly heavily.

Acid phosphatase was only demonstrated when the whole hindgut was incubated and, even under these conditions, staining was far less pronounced than in the midgut. Uniform, moderate activity was demonstrated in the anterior hindgut and along one side of the central hindgut. The short posterior hindgut showed comparatively little activity. In the central hindgut moderate activity was observed in the strip of *A* cells (Plate 2, Fig. 4). The cuticular lining and the basement membrane of the *A* cells together with the investing muscles, all stained quite distinctly. At times weak cytoplasmic staining and scattered black or brown granules were observed adjacent to the cuticular lining. Nuclei sometimes gave a positive reaction. On the other hand, the strip of *B* cells typically failed to react, although there was sometimes some staining of the investing muscles, and particularly those adjacent to the *A* cells.

(viii) *Dehydrogenases*.—The neotetrazolium chloride (NTC) test for dehydrogenase enzymes resulted in one side of the central hindgut staining strongly with deep purple formazan (reduced NTC), whereas the other side only attained a light pink coloration.

Frozen sections demonstrated that the *A* cells contained numerous purple granules, generally evenly distributed throughout the cytoplasm, but occasionally concentrated in the apical region (Plate 2, Fig. 6). On the other hand, the *B* and *C* cells were colourless or stained a light pink and contained very few scattered granules.

Fresh hindguts reduced NTC without added substrate. However, when the hindguts were frozen in saline and thawed, endogenous substrate was lost from the tissues and colour was formed only in the presence of added succinate (0.05M). Lactate, glycerophosphate, malate, fumarate, and citrate at 0.05M were not effective substrates. Deposition of formazan in the presence of succinate was partially inhibited by malonate (0.1M) and completely inhibited by monoiodoacetate (0.025M).

(ix) *Acetyl Esterase*.—A strong positive reaction for acetyl esterase was obtained on one side only of the hindgut, the other side reacting very weakly or not at all. As with the test for dehydrogenases, the heavy staining occurred in the band of A cells. In frozen sections it could be seen that the basal cytoplasm of these A cells stained a deep pink and contained numerous small red granules, whereas the apical half of the cells stained rather less heavily but, at times, contained numerous, rather larger deep red granules (Plate 2, Fig. 3). The B and C cells were generally unstained and only occasionally became light pink (Plate 2, Figs. 3 and 5). These cells rarely contained a few scattered red granules.

(x) *Phenolsulphatase*.—Very weak phenolsulphatase activity (in the form of a diffuse light pink coloration) was observed in the hindgut after long incubation periods, but there was no indication of restriction to any particular cell type.

(xi) *Cytochrome Oxidase*.—The entire hindgut stained rapidly and apparently uniformly with the Nadi reagent for cytochrome oxidase although it is possible that the strong reaction in the musculature might have obscured a difference in the A and B bands. In mounted preparations the staining was seen to be caused to a large extent by small blue granules scattered throughout the cells.

(xii) *Hydrogen Ion Concentration*.—Saturated solutions of sulphonphthalein indicators* in saline were injected into the posterior segment of larvae ligatured so that a small loop of the hindgut was enclosed in the anterior segment. This enabled indicator taken up by the hindgut epithelium posterior to the ligature to be observed without the complication of colour in the lumen caused by indicator which had come via the midgut or malpighian tubules. One side of the hindgut epithelium was found to absorb and accumulate the various indicators, whereas the other side only contained traces or none at all. The side which accumulated indicators strongly had a pH of about 7.4 (very faint pink with phenol red and showing the alkaline colours of bromthymol blue, bromcresol purple, and bromcresol green). The other side appeared on the basis of the very weak colours observed to be rather more acid, perhaps a pH of about 6.4 (a light yellowish green with bromthymol blue, turning light blue on addition of alkali). When indicator-stained guts were tested with the Nessler reagent the principal reaction for ammonia appeared in the lightly stained cells. The alkaline cells are, therefore, presumably the A cells.

(xiii) *Negative Tests*.—No ionizable iron, copper, or zinc was detected in the hindgut epithelium. Tests were also negative for inorganic phosphate, uric acid, ascorbic acid (even after feeding on ascorbic acid-enriched food), lipase, and amine oxidase (which occurs in Crustacea (Blaschko and Himms 1954)).

(c) *Comparison with Other Dipterous Larvae*

A brief examination of the larvae of several other blowfly species (*Lucilia sericata* (Meig.), *Calliphora stygia* (F.), *C. augur* (F.)), of the housefly (*Musca*

* BDH water soluble indicators.

domestica L.) and of *Drosophila melanogaster* Meig. indicated that, in these species also, the hindgut epithelium is histologically and functionally differentiated into longitudinal bands of cells. *L. cuprina*, therefore, is not unique in this respect.

IV. DISCUSSION

A striking feature of the present investigation was the finding that, for the greater part of its length, the hindgut of blowfly larvae is differentiated into longitudinal bands, each formed by a single cell type. This kind of differen-

TABLE 2

TYPICAL REACTION OF *L. CUPRINA* HINDGUT TO VARIOUS HISTOCHEMICAL TESTS

A blank or — indicates a negative reaction, \pm indicates a very weak reaction, and + to +++ indicates increasing intensity of reaction

Histochemical Test	Anterior Hindgut	Central Hindgut			Posterior Hindgut
		A Cells	B Cells	C Cells	
Potassium		++	\pm	\pm	
Barium			+	\pm to ++	
Ammonia		— to +	+++	+++	
Acid phosphatase	+	++	\pm	\pm	\pm
Dehydrogenases	— to \pm	+++	— to \pm	— to \pm	— to \pm
Acetyl esterase		+++	— to \pm	— or \pm	
pH injections		about 7.4	? about 6.4		

No Reaction in Hindgut for:		Weak Uniform Reaction in Hindgut for:	
Iron Copper Zinc Strontium Phosphate Uric acid Ascorbic acid Lipoid spheres Amine oxidase Lipase		“Ground” lipid Glycogen Alkaline phosphatase Phenol sulphatase	
		Strong Uniform Reaction in Hindgut for:	
		Cytochrome oxidase	

tiation does not appear to have been recorded previously and is quite different from the typical successive zones of different morphology, histology, and function into which the insect gut can normally be divided.

Table 2 summarizes the results of the various histochemical tests performed. There is no doubt that the three types of cell producing the longitudinal bands each behave in a distinct fashion, although it is not yet possible to define their differing functions in any detail.

The observations in the present paper enable a clearer picture to be obtained of the route of ammonia excretion in blowfly larvae. When larvae are fed on fresh food, proteolytic enzymes in the digestive tract lumen carry out their degradation without appreciable ammonia production. Ammonia is, however, produced from the partially split products, but only after these have been absorbed by the midgut epithelium (Brown 1938; Lennox 1941*b*). The midgut gives a weak Nessler reaction, possibly because the acidity of its contents results in a greater ammonia capacity than that of the remaining, alkaline regions of the midgut. However, the posterior midgut cells appear to possess the greatest deaminase activity (Lennox 1941*b*).

The ammonia produced appears not to pass into the midgut lumen but out into the haemolymph which contains the surprisingly high concentration of 12 mg ammonia-N per 100 ml (Lennox 1941*a*). Some of this ammonia is absorbed by the malpighian tubules and is presumably discharged into the hindgut or bound as magnesium ammonium phosphate (Waterhouse 1950). However, the bulk of the ammonia is probably taken up directly from the haemolymph by the central hindgut. Two of the three types of epithelial cell present appear to be specially involved in this process.

Carbon dioxide elimination is linked, in part, with ammonia excretion. Tests indicated that regions in the gut of high ammonia and high carbonate coincided. Ammonia liberated by deaminase action may be pictured as passing from the haemolymph to the hindgut lumen where it is associated with bicarbonate. The presence in the excreta of the relatively unstable bicarbonate (rather than of other more stable ammonium salts) would account for the strong ammoniacal odour characteristic of growing blowfly larvae. The binding of carbon dioxide in the excreta would also explain the extremely low respiratory quotient of *Lucilia* larvae (Brown 1938). It may be that the uptake of respiratory carbon dioxide by the hindgut is largely pH-controlled so that, as the pH of the cell and gut contents increases, the rate of carbon dioxide uptake (or retention) is increased due to a change in the carbon dioxide concentration gradient to the tracheal system.

It should be noted that the binding of carbon dioxide in the excreta and a low respiratory quotient from this source is only possible when the deaminated amino acid is either completely oxidized to carbon dioxide and water or, and rather unlikely, converted to a neutral substance such as an alcohol or an aldehyde. This follows from the fact that at a pH of 7.0-7.5 deamination of an amino acid would have little effect on the pH, but that complete oxidation of the fatty acid (which results in the production of several equivalents of carbon dioxide for each equivalent of ammonia produced by deamination) would cause an increase in pH if an appreciable amount of the carbon dioxide produced was lost, for example, by diffusion into the tracheal system.

When, instead of fresh food, food contaminated by excreta is ingested, the crop may contain a high concentration of ammonium bicarbonate, but this is apparently transferred into the haemolymph when the food reaches the anterior midgut. It has been claimed that blowfly larvae are able to excrete their

nitrogen as ammonia, because, like fish, they are provided with an aqueous environment, and because a copious supply of water is necessary for any organism which excretes ammonia (Lennox 1941a; Baldwin 1949). However, this is certainly not the whole story, since blowfly larvae live perfectly well in culture medium into which a great deal of ammonium bicarbonate has been excreted. It appears rather that they are able not only to tolerate relatively high concentrations of ammonia in their haemolymph and food, but also to eliminate it effectively by the activity of portion of their hindgut epithelium. Many aquatic insects excrete ammonia, but evidence so far available suggests that blowfly larvae may be unusual in the concentration that they can tolerate in their haemolymph (Staddon 1955).

No functional relationship can be suggested for the virtual restriction of the reactions for potassium, acid phosphatase, dehydrogenases, and acetyl esterase to the band of A cells, unless it is that the function of ammonia excretion by the B and C cells precludes these from many other activities. If, as appears probable from injection studies, the general cytoplasm of the A cells has a pH of about 7.4, it is perhaps not surprising that the acid phosphatase reaction is confined to the wide inner cuticular lining and to the base of the cells. In the mid midgut, acid phosphatase and dehydrogenases occur in different cell types (Waterhouse and Stay 1955). On the other hand, acetyl esterase and dehydrogenase activity is associated in the same cell type in both regions (Waterhouse, unpublished data).

The significance of the presence of acetyl esterase in the hindgut is not clear, although it occurs widely in many animal tissues and has been recorded in extracts of a number of insects (Lord and Potter 1953, 1954).

No evidence was obtained that the hindgut of blowfly larvae plays the important part in water economy that it does in many other insects. It is interesting to note, too, that a good deal of the protease secreted into the midgut lumen passes out to the exterior through the hindgut, apparently without appreciable destruction or absorption (Hobson 1931; Brown and Farber 1936). However, too little is known of the enzyme content of insect excreta to indicate clearly how unusual this may be.

There is little doubt from the evidence presented that the hindgut epithelium of blowfly larvae is an active and highly differentiated tissue which plays an important role both in intermediary metabolism and in regulating the products of excretion.

V. ACKNOWLEDGMENT

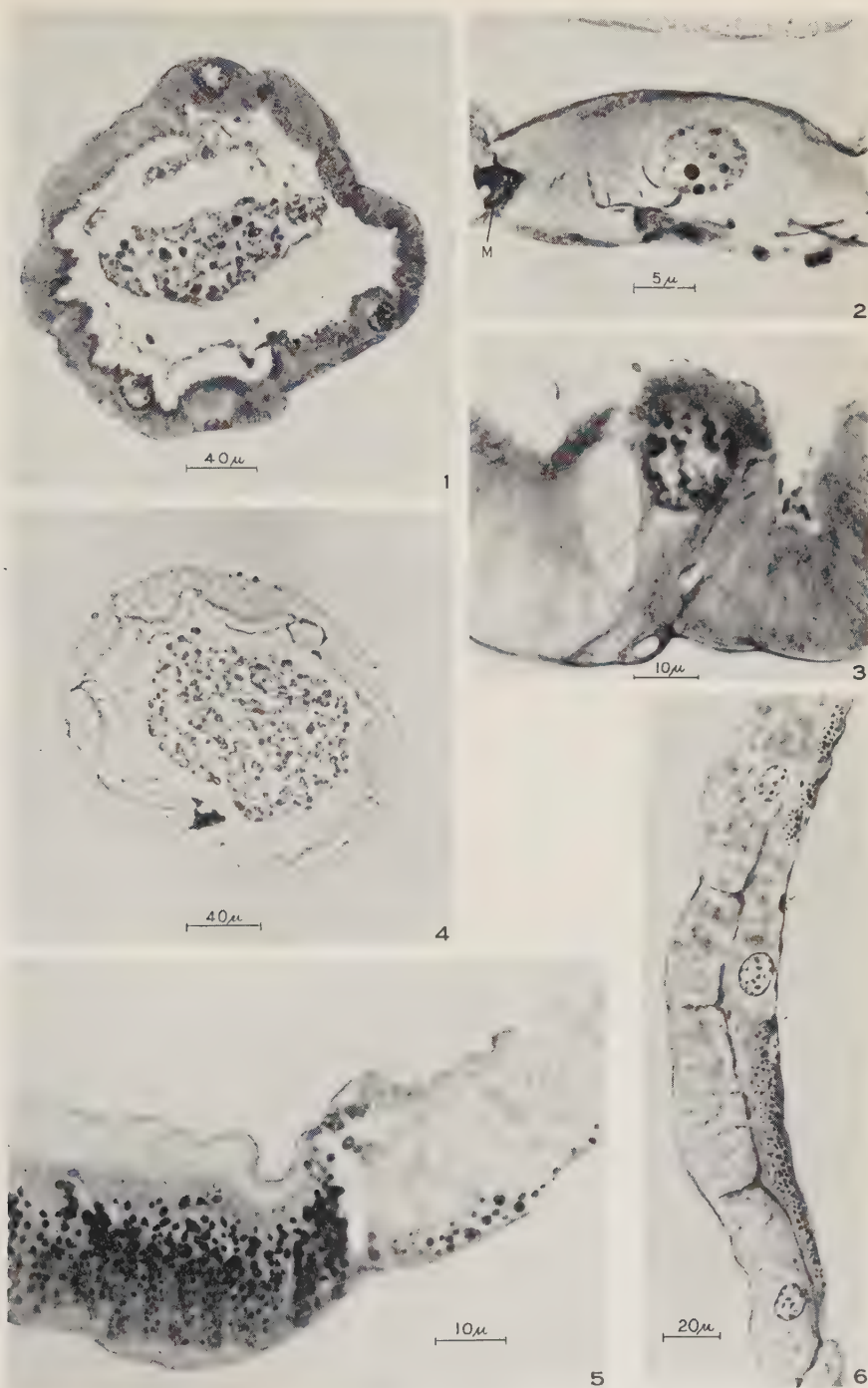
Thanks are due to Dr. B. A. Stay who participated in some of the early observations which led to this paper.

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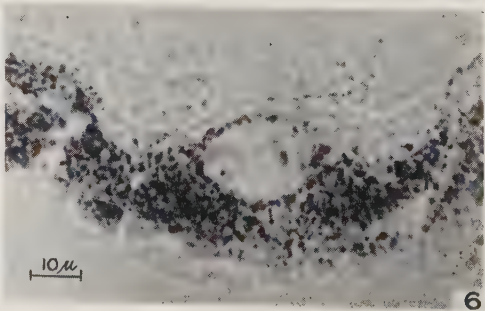
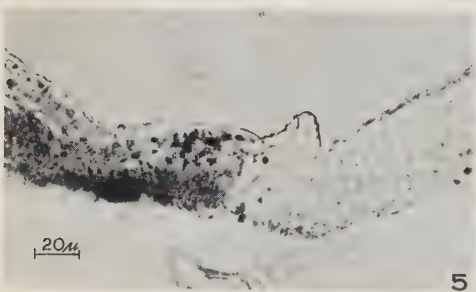
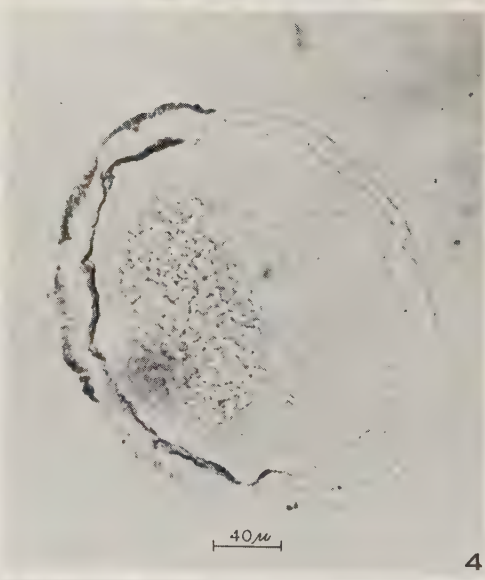
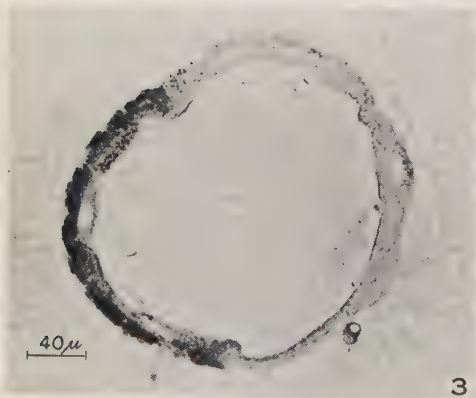
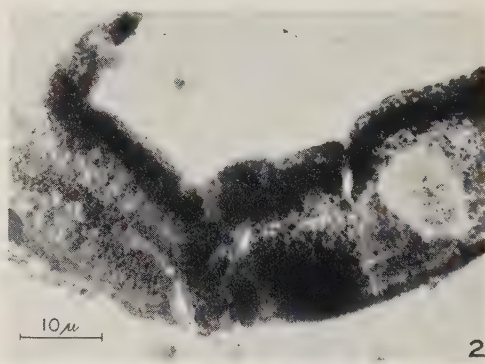
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FUNCTIONAL DIFFERENTIATION OF BLOWFLY HINDGUT



FUNCTIONAL DIFFERENTIATION OF BLOWFLY HINDGUT



EXPLANATION OF PLATES 1 AND 2

PLATE 1

Histology, Potassium, Barium

- Fig. 1.—Delafield's haematoxylin and eosin after alcoholic Bouin's fixation. 10μ cross section of central hindgut showing *A* cells with apical nucleus and wide cuticular lining and *B* cells with median nuclei and narrow cuticular lining.
- Fig. 2.—Bodian's silver protargol after alcoholic Bouin's fixation. 10μ longitudinal section of anterior hindgut showing intracellular tracheae and circular muscles (*M*) invaginated into cells.
- Fig. 3.—As for Figure 1, showing *C* cell with nucleus lying between *A* cell (left) and *B* cell (right).
- Fig. 4.—Neutral formalin fixation, rhodizonate test for barium. 10μ cross section of central hindgut showing positive reaction (black) in *C* cell at 7 o'clock and negative reaction elsewhere, including the *C* cell at 1 o'clock.
- Fig. 5.—Retouched photograph of Macallum test for potassium in central hindgut, indicating intense reaction (black) in the *A* cell on left and weaker reaction in *C* and *B* cells (centre and right).
- Fig. 6.—As for Figure 4. Retouched photograph of longitudinal section of central hindgut, showing *C* cell (right) containing granules stained for barium and *A* cells (left).

PLATE 2

All photographs are cross sections of the central hindgut.

Reactions for Ammonia, Acetyl Esterase, Acid Phosphatase, and Dehydrogenases.

- Fig. 1.—Nessler test for ammonia. 10μ paraffin section showing the main reaction in the vicinity of the *C* cells and positive, but weaker, reaction apically and basally in the band of *B* cells.
- Fig. 2.—As for Figure 1 showing the distribution of the Nessler reaction for ammonia in two heavily stained *B* cells.
- Fig. 3.—Esterase demonstrated with β -naphthyl acetate and Fast Blue RR. Formalin fixation. 10μ gelatin section showing intense esterase activity restricted to the band of *A* cells.
- Fig. 4.—Gomori's acid phosphatase (modified, see text). Note restriction of reaction to apex and base of *A* cells.
- Fig. 5.—As for Figure 3 showing esterase reaction in *A* cell (left) and absence of reaction in *B* and *C* cell (right and centre). Note large granules in apical half and uniform positive ground coloration in basal half.
- Fig. 6.—Dehydrogenases demonstrated with NTC. Formalin fixation. 10μ gelatin section showing *A* cells only. Formazan (reduced NTC) is deposited in large granules in the apex of left cell and generally throughout the cytoplasm of both cells.

STUDIES ON CHITIN

III. ADSORPTION OF PROTEINS TO CHITIN.

By R. H. HACKMAN*

[Manuscript received April 26, 1955]

Summary

The effects of pH, salt concentration, and temperature on the adsorption of a water-soluble insect cuticular protein to chitin have been investigated. The adsorption is dependent upon pH, decreasing rapidly as the pH increases from the region of the isoelectric point of the protein. Increase in salt concentration decreases adsorption but the adsorption appears to be little influenced by changes in temperature. Tyrosine-rich protein fractions are preferentially adsorbed. The adsorption is partly irreversible and an increase to pH 9 is necessary before all the adsorbed protein can be removed. It is concluded that there is only a weak bonding between the chitin and the water-soluble cuticular protein.

I. INTRODUCTION

In an earlier paper (Hackman 1955) the reaction between *N*-acetyl-D-glucosamine (the recurring chemical unit of chitin) and amino acids, peptides, and proteins was investigated. It was shown that compounds of the Schiff base (or azomethine) type are formed but no reaction occurs under acidic conditions. The compounds are unstable and undergo hydrolysis even in the biological pH range. Tyrosine residues, as well as free α -amino groups, were shown to play an important part in the binding of *N*-acetyl-D-glucosamine by insect cuticular proteins.

This evidence supports the theory that in soft insect cuticles there is a weak bonding between chitin and protein. To obtain further evidence along these lines the adsorption of a water-soluble cuticular protein to chitin has been investigated. The information gained must also be useful in further studies on the proteins of insect cuticles.

II. EXPERIMENTAL

(a) Reagents

The protein used was the water-soluble protein extracted from larval cuticles of *Diaphonia dorsalis* Don. (Coleoptera) prepared according to the method described by Hackman (1953). The protein contained 13.9 per cent. N (micro-Kjeldahl). The protein preparation was subjected to electrophoresis on filter paper using an E.E.L. paper electrophoresis apparatus. A phosphate buffer

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(0.100M potassium dihydrogen phosphate + 0.0024M potassium hydroxide, pH 5.3, ionic strength 0.1) and a current of 0.4 mA/cm width of paper were used, and the duration of the experiment was 24 hr. The protein was separated into five components.

The chitin was prepared from lobster shell by the method described by Hackman (1954). To purify the chitin further it was ground in an Eppenbach colloid mill, model QV6. The majority of the particles were below 5μ in their greatest dimension and almost all the particles were less than 10μ . However, there was present an occasional larger particle of up to 30μ in its greatest dimension. The finely ground material was again extracted with cold 2N aqueous hydrochloric acid and hot N aqueous sodium hydroxide. It was finally dialysed against running water until free of alkali. Since the chitin was found to cake on drying it was retained as a suspension. The suspension was quite stable for the duration of these experiments and 2.55 ml contained 50 mg chitin and 2.5 ml water.

Except for the experiment described in Section II (e), all experiments were conducted in a constant temperature room at $26.7 \pm 0.2^\circ\text{C}$.

(b) Effect of pH on Adsorption of Protein

A protein solution was prepared for each pH value in an appropriate buffer solution so that 1 ml contained 2 mg protein. Protein solution (2.5 ml) and chitin suspension (2.55 ml) were mixed well by a gentle rocking motion so as to avoid frothing and then rotated gently for 60 min. The rate of rotation, which was constant for all experiments, was sufficient to keep the suspension well mixed without any frothing. Previous experiments had shown that under the varied experimental conditions described in this paper adsorption was complete within 60 min. The final solutions all had ionic strengths of 0.1 and the pH of the solutions as measured by the glass electrode were 5.05, 6.00, 7.00, 8.09, 8.95, and 9.97. They had the following composition: (i) 0.045M acetic acid + 0.1M sodium acetate, (ii) 0.07M sodium dihydrogen phosphate + 0.01M disodium hydrogen phosphate, (iii) 0.019M sodium dihydrogen phosphate + 0.027M disodium hydrogen phosphate, (iv) 0.0017M sodium dihydrogen phosphate + 0.0317M disodium hydrogen phosphate, (v) 0.09M glycine in 0.09M sodium chloride + 0.01M sodium hydroxide, and (vi) 0.06M glycine in 0.06M sodium chloride + 0.04M sodium hydroxide.

After the suspensions had been rotated for 60 min they were centrifuged. Aqueous sodium hydroxide (0.5 ml of an appropriate concentration) was added to 3.0 ml of the clear supernatants to make the sodium hydroxide concentration 0.75N. The solutions were mixed well and the optical densities read at 280 and $294.4m\mu$ in a Beckman photoelectric spectrophotometer, model DU. The amount of protein in 3.0 ml of these alkaline solutions was also estimated by the biuret method as given by Robinson and Hogden (1940). The optical densities of the coloured solutions were read at $550m\mu$. The amount of protein present in each solution was determined by reference to calibration curves prepared by dissolving the protein in 0.75N sodium hydroxide and (1) measuring the optical density at $280m\mu$, (2) measuring the optical density of the colour

formed in the biuret method. For every pH value solutions were prepared which contained protein but no chitin, and these served as controls. All experiments were performed at least in duplicate and the amount of protein adsorbed by the chitin was determined by difference.

(c) Adsorption Isotherms

The experiments were carried out in a similar manner to those described in Section II (b). Protein solution (2.5 ml) was added to various chitin suspensions (2.55 ml, 2 ml + 0.54 ml water, 1 ml + 1.52 ml water, 0.5 ml + 2.01 ml water, and 0.25 ml + 2.255 ml water). This gave suspensions containing 50, 40, 20, 10, and 5 mg chitin in 5 ml total volume. The solutions all had ionic strengths of 0.1 and two sets of experiments were performed, one at pH 6.00 and the other at pH 7.00 using the buffer solutions described above. The protein present in the supernatant after adsorption, was estimated by the biuret method.

(d) Effect of Salt Concentration on Adsorption of Protein

Three salt solutions were used. The protein (2 mg/ml) was dissolved in the salt solution and 2.5 ml of each solution were added to 2.55 ml chitin suspension. The final salt solutions had the following compositions: 0.019M sodium dihydrogen phosphate + 0.027M disodium hydrogen phosphate, ionic strength 0.1; the above phosphates + 0.05M sodium sulphate, ionic strength 0.25; the above phosphates + 0.15M sodium sulphate, ionic strength 0.55. The pH of all salt solutions was 7.00. The experiment was completed as described in Section II (b).

(e) Effect of Temperature on Adsorption of Protein

The experiments described in Section II (b) for pH 6.00 and 7.00 were repeated at 4°C, and the protein remaining in solution estimated by the biuret method.

(f) Reversibility of the Adsorption

(i) Cuticular protein (5.0 mg) was dissolved in a phosphate buffer of pH 7.00 (2.5 ml) and chitin suspension (2.55 ml) added. Ionic strength of the final solution was 0.1. The suspension was mixed well, rotated gently for 60 min, centrifuged, and the protein in the supernatant estimated by determining its optical density at 280 m μ . Phosphate buffer (3 ml, pH 7.00, ionic strength 0.1) was added to the chitin residue, the suspension mixed well, rotated for 60 min, centrifuged, and the protein in the supernatant estimated as before. Buffer was again added to the chitin residue and the extraction procedure repeated six times in all when no further protein was extracted.

(ii) The cuticular protein was adsorbed on to the chitin from a phosphate buffer of pH 7.00 as in Section (f) (i), but a glycine-sodium hydroxide buffer (pH 8.95, ionic strength 0.1) was added to the chitin residue instead of buffer of pH 7.00. Before adding the buffer solution the pH of the chitin residue was

adjusted to 9.00 by the addition of aqueous sodium hydroxide. A single extraction removed all of the protein from the chitin.

III. RESULTS AND DISCUSSION

Earlier work (Hackman 1953) had shown that the water-soluble cuticular protein from *Diaphonia dorsalis* was not homogeneous but that it contained a number of components. Paper electrophoresis has shown that the protein can be separated into five components. It follows that the methods used to estimate the protein concentrations had to be such that they did not distinguish between the various protein fractions, or if they did then the nature of the discrimination would have to be known. Two methods were chosen: the development of the biuret colour which is almost independent of the nature of the protein, and measurement of the optical density of the protein solution at 280 m μ . The

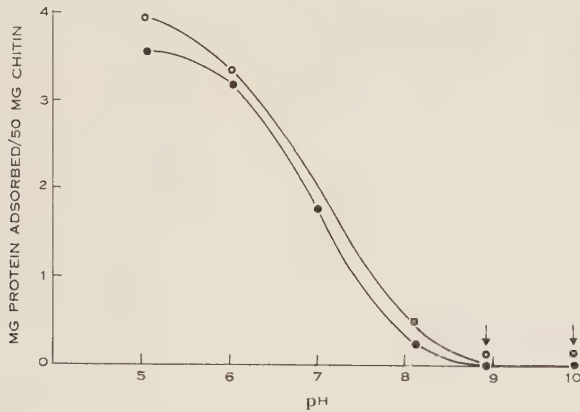


Fig. 1.—Effect of pH on the amount of protein adsorbed onto chitin from an 0.1 per cent. solution of the protein.
● Estimated by the biuret method. ○ Estimated by optical density at 280 m μ .

latter method is dependent upon tyrosine content and such measurements might indicate the adsorption of tyrosine-rich or tyrosine-poor fractions. Reproducibility of the biuret method was found to be excellent and a difference of 0.02 mg protein could be readily and consistently estimated. No alteration in the optical density of the protein solution at 280 m μ , in 0.75N sodium hydroxide was observed over a period of approximately one hour (by which time all measurements in any one experiment had been completed). However, changes which occurred within a few minutes would not have been detected. Even though the methods for analysis of the protein were reproducible with a high degree of accuracy, controls were included in all experiments to ensure that no unforeseen errors occurred.

The effect of pH on the amount of protein adsorbed on to chitin is shown in Figure 1. The graphs show clearly that a tyrosine-rich fraction is adsorbed.

Calculation of the tyrosine content of the protein solutions from the optical densities at 280 and 294.4 $m\mu$ (see Goodwin and Morton 1946) again indicates that a tyrosine-rich fraction is adsorbed. As the protein at a concentration of 1 mg/ml is not soluble in buffer solution of ionic strength 0.1 at pH 3 or 4, measurements could not be made in the region of the isoelectric point (pH 3.4) of the protein. The absence of adsorption at pH 9 or 10 is ascribed to the method of preparing the protein. The cuticular protein was prepared by extracting insect cuticles (which contain chitin) with a buffer of pH 9; consequently

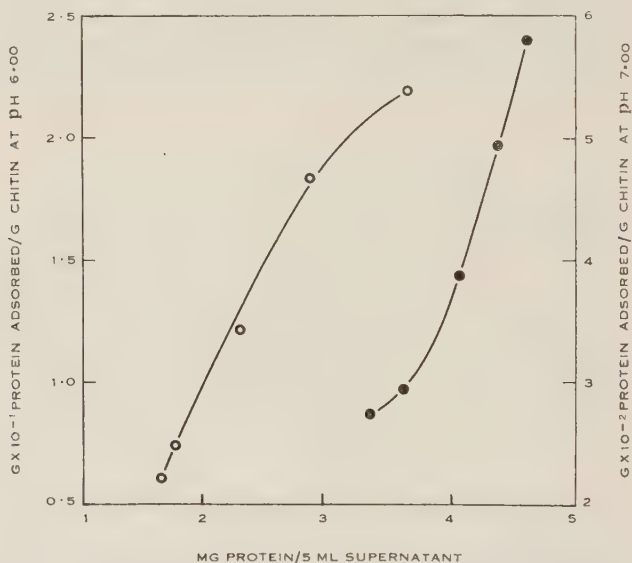


Fig. 2.—Adsorption isotherms obtained by the constant protein method. Protein concentration 0.1 per cent. ● Sodium phosphate buffer (pH 7.00, ionic strength 0.1). ○ Sodium phosphate buffer (pH 6.00, ionic strength 0.1).

no adsorption would be expected at pH 9 or at pH values higher than 9. The effect of pH on the amount of the cuticular protein adsorbed on to chitin is similar to that generally observed for the adsorption of proteins on to solids, i.e. the amount adsorbed decreases as the pH increases from the region of the isoelectric point of the protein.

Adsorption isotherms of proteins may be prepared from data obtained by varying the protein concentration or by varying the amount of adsorbent, i.e. by working with a constant protein or constant adsorbent concentration. If the protein preparation is homogeneous then identical results should be obtained by both methods. The cuticular protein used is known to be non-homogeneous and it has been shown that a tyrosine-rich fraction is preferentially adsorbed. Results obtained by the constant adsorbent method indicated the presence of a

small fraction which was not adsorbed at all; consequently the constant protein concentration method was used to prepare the adsorption isotherms. The results are shown graphically in Figure 2. The slope of the adsorption isotherms indicates that satisfactory elution of the proteins would not be obtained with the same solvents. This fact is considered further when the irreversible nature of the adsorption is discussed.

The effect of salt concentration on the adsorption of the insect cuticular protein on chitin is given in Table 1. An increase in salt concentration brings about a decrease in the amount of protein adsorbed on to the chitin. The protein is not completely soluble at pH 7.00 in solutions of ionic strength greater than 0.55 (i.e. approx. 1/17 sat. sodium sulphate) so the results are of neces-

TABLE 1

EFFECT OF SALT CONCENTRATION ON THE AMOUNT OF PROTEIN ADSORBED ON TO CHITIN FROM 0.1 PER CENT. SOLUTION OF THE PROTEIN AT pH 7.00

Total Ionic Strength	Mg Protein Adsorbed/50 mg Chitin	
	Biuret Method	Optical Density Method (at 280 m μ)
0.10	1.70	2.10
0.25	0.92	1.26
0.55	0.61	0.90

sity limited. Calculation of the tyrosine content of the unadsorbed protein shows a decrease as the ionic strength increases from 0.1 to 0.25 after which it remains constant. Therefore increase in salt concentration at first favours the adsorption of tyrosine-rich protein fractions. In contrast with results produced by changes in salt concentration, temperature had little, if any, effect on the adsorption of protein on to chitin. Experiments carried out at 4°C gave the same results as those carried out at 26.7°C for both pH 6.00 and 7.00.

Adsorption of the insect cuticular protein by finely divided chitin is partly irreversible under the conditions studied. The chitin (50 mg) adsorbed 1.75 mg protein from 5 ml of a buffer solution of pH 7.00 and ionic strength 0.1 which initially contained 1 mg protein/ml. Subsequently by washing the chitin with buffer solution of the same pH and ionic strength, only 0.71 mg (i.e. 40 per cent.) of this adsorbed protein could be removed. It would appear that there is a layer of protein firmly held on to the chitin, and an outer, elutable layer held by secondary forces. However, all the protein adsorbed at pH 7.00 can be removed by extraction with a buffer of pH 8.95 and ionic strength 0.1. Lack of reversibility of adsorption is frequently encountered with proteins, and

in the case of this cuticular protein an increase in pH is necessary before the adsorbed protein can be removed.

In the work described above some of the factors affecting adsorption of an insect cuticular protein to chitin have been investigated. It is quite clear from the results that tyrosine-rich protein fractions are preferentially adsorbed and there is little doubt that the tyrosine residues in the protein play an important role in this adsorption. The adsorption is sensitive to both changes in pH and changes in salt concentration but is largely unaffected by changes in temperature. Since no adsorption occurs at pH 9 and since the protein may be extracted from the chitin at this pH it would appear that neither hydrogen bonds nor covalent bonds are involved in the adsorption (cf. Hackman 1955). This evidence indicates that there is only a weak bonding between the chitin and the water-soluble cuticular protein. The apparent absence of hydrogen bonds is of interest since both the chitin and the protein contain large numbers of groups which could form hydrogen bonds. The application of the results obtained in this paper to a study of insect cuticular proteins will be reported in later papers.

IV. ACKNOWLEDGMENT

The author wishes to acknowledge the helpful advice of Professor A. E. Alexander, N.S.W. University of Technology, Sydney.

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THE AMINO ACID COMPOSITION OF KERATINS

III. THE AMINO ACID COMPOSITION OF DIFFERENT QUALITIES OF WOOL

By D. H. SIMMONDS*

[*Manuscript received April 20, 1955*]

Summary

The amino acid composition of 16-hr 6N HCl hydrolysates of three qualities of commercially classified wools has now been determined using the technique of Moore and Stein (1951). In this paper the results obtained on samples of Merino 70's and Corriedale 56's wool are compared with those previously reported for Merino wool of 64's quality. The overall pattern of the amino acid composition of the three wools is similar although small variations between the wools are observed with some of the amino acids.

I. INTRODUCTION

In Part I of this series (Simmonds 1954*a*), the complete amino acid composition of a 16-hr hydrolysate of Merino wool of 64's quality was presented. In a preliminary survey of the variations to be expected between different qualities of wool, similar analyses have now been performed on two additional samples commercially classified as Corriedale 56's and Merino 70's. The results of these analyses are reported in the present paper.

II. EXPERIMENTAL

(a) Preparation of Wool for Analysis

Samples of virgin Corriedale and Merino wool (5 g) were prepared for analysis as previously described (Simmonds 1954*a*).

(b) Analytical Procedures

The apparatus, solutions, and procedure were also the same as described in Part I, except that accurate collection of 1 ml fractions was achieved by the use of the magnetic balance described by Simmonds (1954*b*). Two or more analytical runs could be carried out simultaneously by arranging the appropriate number of balances around the fraction collector turn-table, each delivering into a separate row of tubes. Only one of the balances was wired to actuate the turn-table, and the column feeding this was adjusted to run slightly faster than the others. In this way double fractions from the subsidiary magnetic balances were avoided. By reducing the total loading on the 100-cm "Dowex 50" columns to 0.3 mg nitrogen, and on the 15-cm columns to 0.5 mg nitrogen,

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the ninhydrin colours of the amino acid peaks were kept below a maximum optical density of 1.25; they could then be accurately read without a secondary dilution, on a "Uvispek"* spectrophotometer.

TABLE 1
AMINO ACID AND ELEMENTARY COMPOSITION OF DIFFERENT WOOLS

Amino acid nitrogen expressed as a percentage of total nitrogen*

Amino Acid and Element	Merino 64's Wool		Merino 70's Wool		Corriedale 56's Wool	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
Alanine	3.51	0.08	3.51	0.15	4.37	0.29
Arginine	20.32	0.04	19.35	0.69	18.21	0.61
Aspartic	4.24	0.18	4.68	0.08	4.86	0.13
Amide N†	7.46	0.57	7.92	0.38	9.27	0.53
Cystine§	7.93	0.05	6.50	0.10	6.80	0.08
Glutamic‡	8.58	0.13	8.54	0.21	9.69	0.33
Glycine	5.80	0.09	6.60	0.16	6.40	0.30
Histidine	1.46	0.10	1.48	0.10	1.59	0.08
Isoleucine	1.97	0.05	2.13	0.07	2.38	0.05
Leucine	4.90	0.12	5.37	0.14	5.51	0.10
Lysine	3.25	0.15	3.19	0.21	3.72	0.09
Methionine§	0.31	0.01	0.37	0.01	0.37	0.01
Phenylalanine	1.75	0.09	2.28	0.20	2.35	0.09
Proline§	5.33	0.02	5.12	0.02	5.52	0.02
Serine†	7.25	0.19	8.63	0.66	7.71	0.16
Threonine†	4.61	0.13	4.12	0.10	4.84	0.15
Tryptophan§	1.73	0.12	1.38	0.06	1.80	0.08
Tyrosine	2.97	0.08	3.09	0.15	3.11	0.18
Valine	3.57	0.10	3.56	0.17	4.50	0.21
Carbon (%)	50.23		50.66		50.65	
Hydrogen (%)	8.13		8.08		7.82	
Nitrogen (%)	16.62		16.57		16.80	
Sulphur (%)	3.68		3.25		3.43	
Ash (%)	0.11		0.22		0.38	

* Amino acid results quoted above represent the means of at least three determinations on each hydrolysate.

† Uncorrected for decomposition of serine and threonine during hydrolysis with 6N HCl.

‡ Corrected for pyrrolidone carboxylic acid formation (Moore and Stein 1951).

§ Determined by methods described in Simmonds (1954a).

Methionine, tryptophan, and proline were estimated by the procedures already described (Simmonds 1954a). Cystine was determined by several procedures, but because of the variability in the results obtained by different

* From Adam Hilger and Co. Ltd., London.

methods, only those given by the method of Folin and Marenzi (1929) and Shinohara (1935, 1937) have been included in Table 1. Investigations on this subject are still in progress, and the results will be reported in a subsequent paper.

III. RESULTS

Table 1 summarizes, in terms of per cent. total nitrogen, the amino acid composition of 16-hr 6N HCl hydrolysates, of one sample each of Corriedale 56's and Merino 70's quality wool, as obtained in the present investigation. The results previously reported for Merino wool of 64's quality (Simmonds 1954a) are included for comparison.

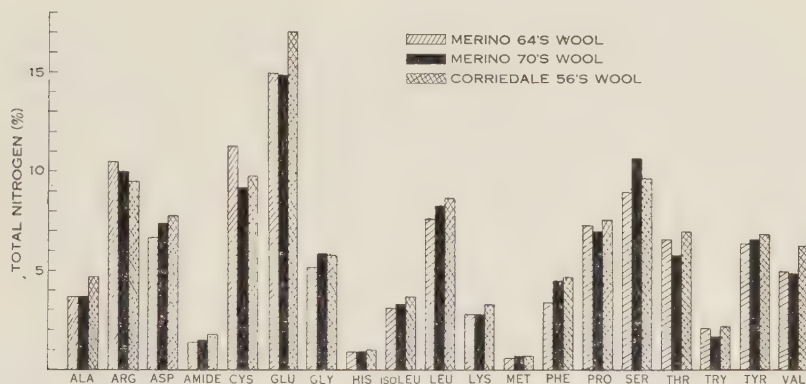


Fig. 1.—Amino acid composition of one hydrolysate from each of Merino 64's, Merino 70's, and Corriedale 56's quality wools.

IV. DISCUSSION

A comparison of the amino acid composition of the three types of wool is rendered easier by reference to the block diagram of Figure 1, where the percentage by weight of each amino acid is presented.

From this it is seen that the overall pattern given by the three samples of wool is similar. In all cases the nitrogen content has been almost quantitatively accounted for, making it unlikely that any further unknown amino acids are present in these hydrolysates.* The two samples of Merino wool differ by more than 10 per cent. in their aspartic acid, cystine, glycine, phenylalanine, serine, and threonine contents, while the Corriedale 56's hydrolysate differed

* Through the kindness of Dr. P. B. Hamilton (A. I. du Pont Institute of the Nemours Foundation, Wilmington 99, Delaware, U.S.A.), we have been able to throw some more light on the nature of the peak labelled Unknown (2) in Figure 2 of Simmonds (1954a). Dr. Hamilton has examined one of our samples which gave an appreciable peak at an effluent volume of 35-40 ml, using the method described by Hamilton and Anderson (1954), and was unable to detect hydroxylysine at a column loading of 20 mg wool (i.e. twice the loading reported by Simmonds 1954a). We have repeated this work in this Laboratory and feel, in agreement with Dr. Hamilton, that the peak labelled Unknown (2) is probably an artefact associated with a change in the eluting buffer from pH 5.0 citrate to pH 6.8 phosphate buffer.

by more than 10 per cent. from the Merino 64's sample in its alanine, arginine, aspartic acid, amide, glutamic acid, glycine, isoleucine, leucine, lysine, phenylalanine, and valine contents. Although the different methods of analysis used indicated different absolute amounts of cystine to be present, the relative amounts in the three wool samples were always the same. The Merino 64's always had the highest cystine content, the Merino 70's the lowest, and the Corriedale 56's was intermediate. It must be emphasized that except for the cystine, methionine, and tryptophan results, the analyses reported are replicate determinations on the same wool hydrolysate, and hence do not include variations from sample to sample. Recent analyses from the Western Regional Research Laboratories, California, U.S.A., reported by von Bergen (1954) and carried out using microbiological methods, showed that the four wools examined (described as New Zealand medium, U.S. medium, Australian fine, and U.S. fine) differed only slightly in their amino acid composition. In view of the variation between the wools reported in the present paper, work at present in progress aims to assess the extent of the variation from site to site on the sheep, between individual sheep of the same strain, and between different strains of Merino sheep. These results will be reported in a subsequent paper, and will be used as a basis for determining differences in amino acid composition between different breeds of sheep.

V. ACKNOWLEDGMENTS

It is a pleasure to record appreciation of the technical assistance rendered by Mr. F. J. Lesock and Mr. I. G. Stell during the course of this work. The author's thanks are also due to Mr. W. B. Hall, Division of Mathematical Statistics, C.S.I.R.O., for helpful criticism. Dr. K. W. Zimmermann carried out the carbon, hydrogen, ash, and sulphur analyses.

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FUNGAL CELLULASES

IV. PRODUCTION AND PURIFICATION OF AN EXTRACELLULAR β -GLUCOSIDASE OF *STACHYBOTRYS ATRA*

By M. A. JERMYN*

[Manuscript received April 14, 1955]

Summary

The accumulation of β -glucosidase in shake cultures of *Stachybotrys atra* is dependent on a variety of physiological conditions but there is no evidence that it is induced by the presence of β -glucosides in the medium. One strain of the mould requires a factor present in yeast extract for the production of the enzyme.

The enzyme is concentrated by precipitation with ethanol from the crude culture medium. It is then fractionally precipitated from the dialysed concentrate by increasing the pH in the presence of 0.1M lead acetate. The bulk of the associated polysaccharide can be removed from the most active preparations by adsorbing the enzyme on a magnesium carbonate column and eluting it with sodium sulphate solution. A single peak of activity appears in the eluate fractions indicating that there is probably only one enzyme component.

Some of the relevant chemical and physical properties of the enzyme are discussed. It is not a cellobiase or a cellulase.

I. INTRODUCTION

Attempts at deducing the course of the enzymatic hydrolysis of cellulose have usually proceeded by the synthesis of information from a large number of distinct organisms. We prefer to investigate the nature and activities of the complete range of enzymes capable of splitting β -glucosidic linkages that are produced by a single organism. In other papers of this series, Jermyn (1953a) has shown that *Stachybotrys atra* secretes into the culture medium an enzyme that hydrolyses *p*-nitrophenyl- β -D-glucoside when grown in shake culture on a variety of non-cellulosic substrates, and Thomas (1956) has shown that little or none of this enzyme is secreted during growth on cellulose under conditions where considerable amounts of cellulase are produced. This readily-measured activity in which a β -glucosidic linkage is split by an enzyme other than cellulase has therefore been used as an indicator in the purification of a β -glucosidase, the properties of which will be described in subsequent papers.

II. METHODS

(a) Estimation of β -Glucosidase by Liberation of *p*-Nitrophenol

Three ml of 1.67×10^{-3} M *p*-nitrophenyl- β -D-glucoside, 1 ml of McIlvaine citrate-phosphate buffer (pH 5.0), and 1 ml of enzyme solution are incubated

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for 20 min at 28°C. Two ml of 7.5 per cent. (w/v) K_2HPO_4 solution are added (final pH 8.5) and the optical density at 400 $\text{m}\mu$ measured in a Coleman Universal spectrophotometer, using blanks for the colour due to enzyme and substrate. The plot of enzyme concentration against optical activity is linear to an optical density of 0.45 (0.5 in. light path); alkaline *p*-nitrophenolate solutions are known to show deviations from Beer's law in wide-band spectrophotometers (Goldenberg 1954). It is possible to correct for this by a calibration curve, but it is more accurate in practice to adjust enzyme concentrations so that the final optical density is low.

The unit of β -glucosidase activity is defined as the amount of enzyme which when dissolved in 1 ml gives an optical density of 1.0 under the standard conditions. In practice an enzyme solution that gives an optical density of *D*, has an activity of *D* units per ml.

The same method has been used with *p*-nitrophenyl- β -D-xyloside and *p*-nitrophenyl- β -cellobioside and the *p*-nitrophenyl-D-mannosides as substrates; *m*-nitrophenyl- β -D-glucoside can also be used, but *o*-nitrophenyl- β -D-glucoside, theoretically a more attractive substrate (lower pH for colour development), is scarcely attacked by the *S. atra* enzyme.

(b) Estimation of β -Glucosidase by Phenol Liberation

Jermyn and Thomas (1953) amongst others (Miwa *et al.* 1949; Takano and Miwa 1950; Barker, Bourne, and Stacey 1953; Buston and Jabbar 1954) have shown that the fact that β -glucosidases are also glucotransferases makes it unsafe to use glucose liberation as a measure of the enzymic hydrolysis of phenyl- β -glucosides. A method was therefore devised for measuring phenol liberation based on the A.O.A.C. (1940) method for phosphatase.

Seven ml of a $1.43 \times 10^{-3}\text{M}$ solution of a given phenyl- β -glucoside was incubated with 2 ml of McIlvaine citrate-phosphate buffer and 1 ml of enzyme for 30 min at 28°C, 5 ml of diluted (1 in 3) Folin-Ciocalteu reagent added, followed by 3 ml of 14 per cent. (w/v) Na_2CO_3 solution after 3 min, and the test tube plunged into boiling water for 5 min. After cooling and filtering the contents of the tube, the colour developed was measured in the Coleman Universal spectrophotometer at 700 $\text{m}\mu$ with appropriate blanks for the substrate and enzyme.

Other glycosides of the phenols may be used as substrates in the same way.

(c) Estimation of Cellobiase, Sucrase, Amylase, and Related Activities

To 9 ml of a solution of sucrose (10^{-3}M) or cellobiose ($5 \times 10^{-4}\text{M}$) in diluted pH 5.0 McIlvaine buffer (1 in 10) is added 1 ml of enzyme solution and the mixture incubated for 1 hr at 37°C. One ml is then withdrawn and the reducing sugar estimated by the Somogyi-Nelson colorimetric method (Nelson 1944), with a blank for the reducing power of the enzyme. For sucrose, the total reducing sugar present was expressed as μg of invert sugar by comparison with a standard mixture of glucose and fructose; for cellobiose it was possible to calculate the degree of breakdown from the known reducing

power of cellobiose and glucose, but for most purposes it was sufficient to use the ratio of the increase in optical density above that of a blank containing cellobiose alone to the optical density of the blank as a measure of cellobiase activity.

The enzymic hydrolysis of other oligosaccharides was estimated by the same method.

In the later stages of this study it was found that a 0.1 per cent. solution of starch could be used to estimate amylase by this method, although earlier the method of Crewther and Lennox (1953) had been used; the two methods gave results in agreement. The hydrolysis of other polysaccharides such as laminarin, xylan, inulin, and *Pseudomonas tumefaciens* polysaccharide was studied similarly.

(d) Estimation of Protease

The method employed *p*-dimethylaminobenzeneazocasein (Jermyn 1953*b*) as the substrate. Eight ml of a 1 per cent. solution of the azocasein in sodium phosphate buffer (final pH 7.0) and 1 ml of enzyme solution are incubated for 30 min at 37°C. The protein is precipitated by the addition of 1 ml of N trichloroacetic acid and, after centrifugation, the optical density of the supernatant is measured at 500 m μ . A calibration curve is necessary to convert optical density to enzyme concentration.

(e) Estimation of Esterase

The chlorophenyl red acetate (Jermyn 1953*a*) method was used.

(f) Estimation of Carbohydrate

Anthrone (20 mg) is dissolved in 70 ml of conc. H₂SO₄ and 30 ml of water added. Ten ml of the cooled reagent are added to 1 ml of the solution to be tested which has been adjusted to contain 50-150 μ g of carbohydrate. The tube and its contents are heated in a boiling water-bath for 7.5 min, cooled, and the optical density at 625 m μ measured against a carbohydrate-free blank. A standard containing 100 μ g of a sugar is heated simultaneously.

Outside the concentration ranges indicated there is considerable departure from proportionality between carbohydrate content and optical density. The method is an adaptation of that of Black (1951) and uses the optimal reagent concentration and heating time.

(g) Filter Paper Electrophoresis and Chromatography

The conditions used have been fully set out by Jermyn (1953*b*) and Jermyn and Thomas (1954). The position of β -glucosidase on the paper was shown by spraying with a solution of 6-bromo-2-naphthyl- β -D-glucoside and tetrazotized di-*o*-anisidine (the reagents of Cohen *et al.* 1952) in a buffer solution which would bring the final pH of the paper to about 5. Any active β -glucosidase showed up as a purple area after a short incubation at 37°C; washing the paper in water to remove the reagents left a permanent record of the position of the enzyme.

(h) Shake Culture

The reciprocating shaker and conditions of spore production used in the earlier work (Jermyn 1953a) were used without change.

III. ENZYME PRODUCTION

The object of this study was to examine the properties of the *S. atra* β -glucosidase rather than to track down all the physiological factors concerned in its secretion. Hence it was found convenient not to alter the general con-

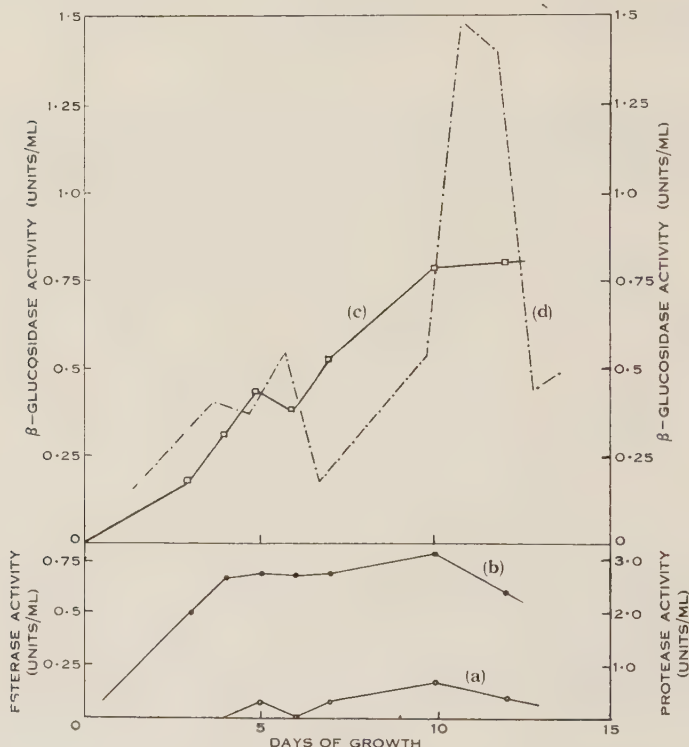


Fig. 1.—Enzyme production by *S. atra* grown on Waksman-Carey medium containing 1 per cent. salicin at 28°C in the continuous recording apparatus. Only those activities relevant to the argument are displayed in this and the following figure. (a) Protease activity. (b) Esterase activity. (c) β -Glucosidase activity. (d) β -Glucosidase activity for a parallel experiment with 1.2 per cent. starch (Jermyn 1953a).

ditions of growth (shake culture in medium-sized flasks, mineral medium (Waksman-Carey), and temperature of operation (28°C)) that were already employed. Only the effects of variations within this framework have been investigated.

(a) Effects of the Carbon Source

S. atra will grow readily on a wide variety of carbon sources (Jermyn 1953a) with a distinct preference for sugars and polysaccharides. When grown on any of the sugars and their polymers so far tested it secretes β -glucosidase. Growth with enzyme secretion has been found to occur on D- and L-arabinose, D-galactose, D-xylose, and maltose as well as the substrates studied in the earlier paper. None of these carbon sources appeared to be as effective as starch in promoting enzyme secretion.

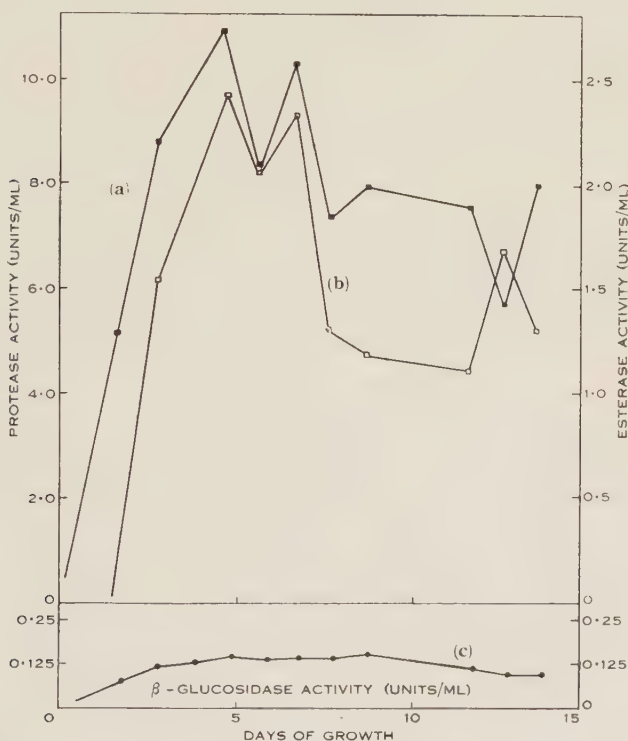


Fig. 2.—Enzyme production by *S. atra* grown on Waksman-Carey medium containing 2 per cent. ground wool topings at 28°C in the continuous recording apparatus. The scale is identical with that of Figure 1. (a) Protease activity. (b) Esterase activity. (c) β -Glucosidase activity.

Since it appeared possible that there might be an “adaptive” effect in the production of the enzyme, growth on carbon sources containing β -glucosidic linkages was studied. The continuous recording apparatus described earlier (Jermyn 1953a) was employed to study growth on a number of such carbon sources.

Figures 1 and 2 show growth curves obtained for growth on salicin (a stable aryl- β -glucoside) and ground wool. In the latter case an apparent adaptive response occurs and protease is secreted into the medium in measur-

able quantities, a result which does not occur with any other carbon source so far tested. There is also a marked increase in esterase activity. Comparison of the salicin curves with those in Jermyn (1953a) for glucose, sucrose, and starch show no demonstrable adaptive response. Similar results were obtained for cellobiose, *p*-nitrophenyl- β -glucoside, and carboxymethylcellulose. Thomas (1956) has demonstrated little or no β -glucosidase production by *S. atra* in the presence of the insoluble polymeric β -glucoside cellulose, even though the mould is at the same time producing a cellulase adaptively.

Since the β -glucosides are rapidly hydrolysed in the culture medium, small quantities in the presence of another carbon source as the major substrate might be effective in inducing increased enzyme production. Salicin and cellobiose (1 per cent. on added glucose) in a 2 per cent. glucose medium did not increase the secretion of β -glucosidase over that on the glucose alone.

It thus appears that the secretion of the β -glucosidase has not been shown to be an adaptive response (no experiments bearing on the intracellular production of the enzyme have yet been carried out). Further work on the production of β -glucosidase was therefore carried out on media containing starch as the carbon source. There appears to be no reason to suppose that the occasional β -glucosidic linkages postulated in native starch by Peat, Thomas, and Whelan (1952) play any part in stimulating β -glucosidase production.

(b) A Factor in Yeast Extract Affecting β -Glucosidase Production by *S. atra*

For two to three years, in the initial stages of these investigations, it was possible to carry out physiological experiments and obtain reliable amounts of enzyme and mycelium from one batch to the next. A change then occurred without apparent reason and only a few flasks in each batch produced enzyme. Finally, enzyme production ceased although growth of the mould was unaffected. It was found that the "degenerated" strain could be induced to produce enzyme when the medium was supplemented with yeast extract (2 g/l).

The properties of the " β -glucosidase factor" which is in no way a "growth factor" will be treated in forthcoming papers by Jermyn and McQuade.

Subsequent to these observations yeast extract was added to all growth media when maximal β -glucosidase production as such was desired.

(c) Physiology of Enzyme Production

The following factors have been shown to exert a consistent influence on the secretion of β -glucosidase by *S. atra* in shake culture:

(i) *Carbon Source.* Rapid growth on a carbohydrate or related substance (glycerol is as satisfactory as glucose) is necessary for the secretion of workable quantities of enzyme. Insoluble substrates such as cellulose and wool (Fig. 2) are unsatisfactory, nor was appreciable activity developed in acetate media.

(ii) *Aeration.* No enzyme secretion is observed during surface culture of *S. atra* on liquid media with glucose as carbon source. A few runs with a small stainless steel fermenter showed that under conditions of relatively vigorous

aeration it was difficult to apply any conclusions drawn from shake-culture experiments; on one occasion an *S. atra* inoculum which, in the absence of added yeast extract, uniformly gave cultures showing no enzyme secretion on the shaker gave a culture with good activity in the fermenter.

To test the effect of aeration in shake culture a factorial experiment was run in which the influences of size of Erlenmeyer flask and volume of medium (Waksman-Carey containing 1 per cent. starch) were compared. The interaction flask-size \times liquid volume had a significant effect on enzyme yield, 100 ml of medium in a 500-ml flask giving optimum results. Such combinations as 50 ml in a 1-l. flask and 150 ml in a 250-ml flask gave little or no enzyme.

(iii) *Mycelial Transfer*. When some of the mycelium from the sporadic flasks containing active enzyme appearing during growth of the degenerated strain on media not containing yeast extract was transferred to a fresh flask, growth was vigorous but no enzyme was secreted. On the other hand, mycelial transfer from an enzymically active culture on a starch medium supplemented with yeast extract to fresh medium of the same type was associated with a heightened production of enzyme. Culture filtrates from *S. atra* grown on starch-yeast extract media (50 ml in 250-ml flasks) for 4-5 days had an enzyme content of 1-2 units/ml. If such cultures were tipped into 700 ml of the same medium in 2-l. flasks, a further 3 days growth gave an activity of 8-10 units/ml. This method was adopted for bulk enzyme production.

(iv) *Duration of Culture*. Jermyn (1953a) showed that the main changes during growth of *S. atra* on sugar solutions in shake culture at 28°C (increase in mycelial weight, fall in pH of the medium) took place within a period of 24 hr, 3-5 days after inoculation with mould conidia. Secretion of β -glucosidase into the medium takes place with similar abruptness when the mould is grown under optimal conditions. In cultures derived from conidial inoculations into starch media this liberation of enzyme usually lags 1-2 days behind the mycelial growth. A number of curves illustrating the time relationship of enzyme secretion and the scatter of results between successive flasks are shown in Figures 3(a) and 3(b). Mycelial growth is more rapid and enzyme secretion occurs earlier in these Erlenmeyer-flask cultures than in the continuous recording apparatus used to obtain the curves figured by Jermyn (1953a).

After the maximum level of enzyme activity is reached the subsequent fall takes a variable course in different cultures. This is at least partly due to small differences in the final pH of the medium, since the stability of the enzyme falls sharply in the pH range 4.0-3.0.

On yeast-extract medium the degenerated strain shows less variability than did the original strain on the basal medium. Figures 3(a) and 3(b) are derived from the latter type of experiment.

Cultures initiated by mycelial transfer from an enzymically active culture to a medium supplemented with yeast extract normally show simultaneous growth and enzyme production.

In a series of experiments certain other factors have been eliminated from consideration as factors influencing enzyme production. These include:

(1) Variations in the procedure for making up the medium such as dissolving the starch before autoclaving and altering the brand of soluble starch employed.

(2) Number of conidia in the inoculum. A certain minimum number of spores (10^2 - 10^3) seems necessary to initiate growth in shake culture (50 ml in 250-ml flasks), but increasing the number of spores has no effect on the final enzyme activity, although it leads to an earlier maximum.

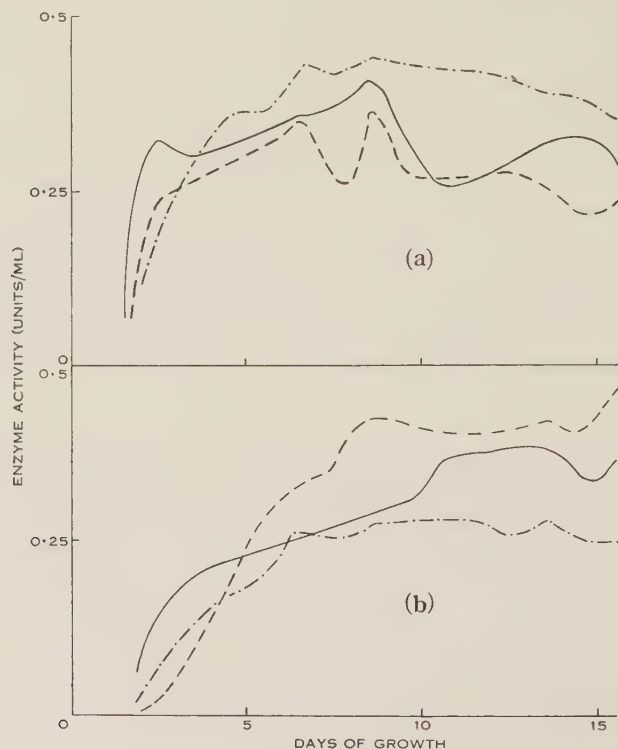


Fig. 3.—Variation in β -glucosidase secretion by *S. atra* grown in shake-culture flasks under identical conditions (yeast extract absent, 100 ml of 1 per cent. starch medium in 500-ml Erlenmeyer flasks at 28°C). Pairs of flasks were inoculated on 3 successive days (solid, dot-dash, and dashed lines) with fresh batches of conidia (*c.* 10^8 per flask). The curve for one member of each pair is given in (a) and (b).

(3) Batch to batch variations associated with different lots of inoculum. It could not be shown that different conidial inocula from the "undegenerated" strain gave significantly different yields of enzyme. Nor does the degenerated strain show significant variation from batch to batch in the presence of yeast extract. Only during the period of transition did successive inoculations give conflicting results.

IV. PREPARATION OF CULTURE FILTRATES

All experiments on the large-scale purification of *S. atra* β -glucosidase have been done with cultures initiated by mycelial transfer between media supplemented by yeast extract. A 250-ml flask containing 50 ml of medium (Waksman-Carey, 1 per cent. starch by weight, 0.2 per cent. yeast extract by weight) was inoculated with about 10^6 conidia and shaken for 4 days at 28°C. The contents were then tipped into 700 ml of the same medium in a 2-l. flask and shaken until β -glucosidase activity reached a maximum (about 3 days).

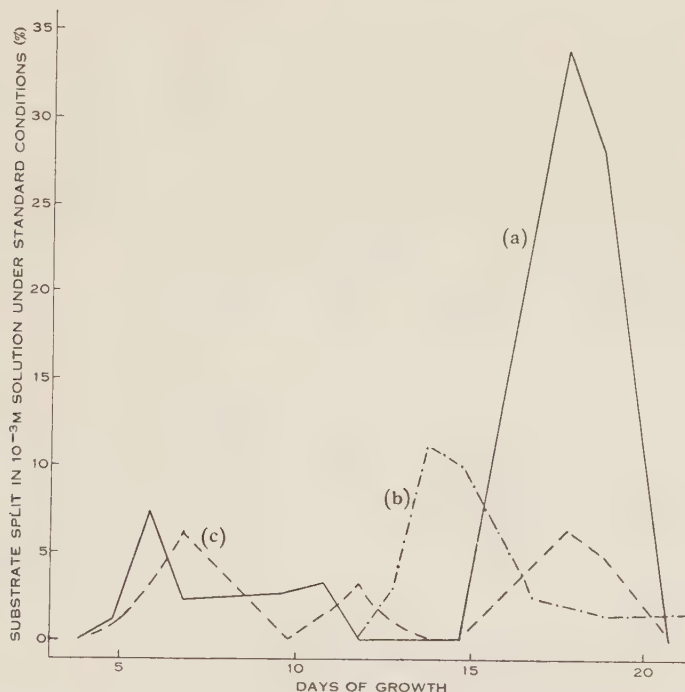


Fig. 4.—Secretion of various enzymes by *S. atra* grown on 2 per cent. glycerol medium in the continuous recorder. (a) Cellobiase. (b) *p*-Nitrophenyl- β -glucosidase. (c) Sucrase.

The contents of the flasks were filtered off through cheese cloth and the compacted mycelium washed with about 1/5 volume of water. The pooled filtrates could be stored at 1°C for an indefinite period without loss of activity.

The bulked mycelium was washed with water till the washings were devoid of β -glucosidase activity and then stirred into 10 volumes of acetone at room temperature. The acetone-extracted mycelium was filtered off, beaten up with further acetone in a Waring Blendor for a short period, centrifuged, and washed with several changes of acetone. The final air-dried powder was stable over a long period and various enzymic activities could be extracted from it after storage for a year at room temperature.

TABLE 1
ENZYMIC ACTIVITIES OF *S. ATRA* GROWN ON STARCH MEDIA

The mycelial extract of Batch B was prepared by ball-milling the acetone-dried mycelium with two parts of silica flour and extracting the resultant powder with water equivalent to the original volume of medium. The activities of the glycosidases are expressed as percentage of enzymically susceptible bonds split in a 10^{-3} M solution of the substrate under the standard conditions

Enzymes and Substrates	Enzyme Activity		
	Batch A Culture Filtrate	Batch B	
		Culture Filtrate	Mycelial Extract
Phosphatase			
Acid	Nil		
Alkaline	Nil		
Protease	Nil		
Esterase	Trace		
Catalase	Negligible		
Laminarinase	Nil	Nil	0.25
Xylanase	Nil		
Amylase	Nil	0.16	0.10
Glycosidase substrates			
Maltose	12.4		
Cellobiose	Nil	6.9	34.9
Lactose	Nil		
Turanose	Nil		
Mellibiose	Nil		
Sucrose	213.0	74.8	80.8
Trehalose	1.0		
Phenyl- α -galactoside	0.59		
2-Naphthyl- β -galactoside	0.48	31.9	46.7
Menthyl- β -glucuronide	1.0		
<p>-Nitrophenyl- β-xyloside</p>	0.36		
<p>-Cresyl- β-glucoside</p>	24.3		
<p>-Cresyl- β-glucoside</p>	5.8		
Thymyl- β -glucoside	3.6	2.0	9.6
Phenyl- β -glucoside	16.8	23.5	41.5
<p>-Chlorophenyl- β-glucoside</p>	25.1		
2-Naphthyl- β -glucoside	28.3		
6-Bromo-2-naphthyl- β -glucoside	43.7		
Salicin	4.9	5.2	21.0
<p>-Nitrophenyl- β-glucoside</p>	17.7	25.6	28.1
<p>-Nitrophenyl- β-glucoside</p>	11.9		
<p>-Nitrophenyl- β-glucoside</p>	Nil		
Methyl- β -glucoside	2.0		
<p>-Nitrophenyl-α-glucoside</p>	0.17		
Methyl- α -glucoside	2.3		
Viscometric cellulase (Thomas 1956)	Nil*		

* After concentration of the culture filtrate 35 times by vacuum evaporation, the enzyme could just be detected.

V. ENZYMIC ACTIVITIES OF THE CULTURE FILTRATE AND MYCELIUM

Although the culture filtrate was looked on primarily as a source of β -glucosidase, the other enzymic activities present were investigated since their presence or absence at later stages in the concentration of the β -glucosidase was one index of the purification of the enzyme. In Table 1 are set out results for two batches of culture filtrate and an extract of the mycelium of one of them.

The sporadic appearance of cellobiase activity in the culture medium and its consistent presence in the mycelium are features of all batches investigated. The secretion of a β -glucosidase into the medium by *S. atra* which does not hydrolyse cellobiose is illustrated in Figure 4 where the peak of *p*-nitrophenyl- β -glucosidase activity comes at a point where there is no cellobiase in the medium and the peak activity of the latter comes some days afterwards.

There is evidence from a number of experiments (cf. Table 1) that preparations with strong cellobiase activity show a much higher salicinase to *p*-nitrophenyl- β -glucosidase ratio than preparations without cellobiase. Hence it is probable that "cellobiase" is in fact one or more β -glucosidases with different specificities from the one investigated in this work. In the absence of cellobiase the ease of hydrolysis of various β -glucosides always follows a fixed order which is the same as that deduced from the specificity properties of the enzyme.

Other activities which appear from time to time in the culture medium are laminarinase (always in association with cellobiase), xylanase, and amylase. Preparations hydrolysing laminarin (poly-1,3- β -D-glucose) do not affect *Pseudomonas tumefaciens* polysaccharide (poly-1,2- β -D-glucose). β -Xylosidase and β -cellobiosidase will be discussed in Part VI of this series in connection with the specificity of the β -glucosidase.

VI. PURIFICATION OF THE β -GLUCOSIDASE

(a) Concentration of the Enzyme

Below 40°C and at neutral or slightly alkaline pH, the β -glucosidase is stable for long periods. Culture filtrates can therefore be concentrated readily in a climbing film evaporator to about 1/20 volume with little loss of activity. Such concentrates are rather viscous and cannot be dialysed since insoluble, brown, gel-like material is precipitated on which the enzyme appears to be irreversibly absorbed. This material can be removed by three successive precipitations of the enzyme with two volumes of ethanol, either in highly-coloured supernatants or as a precipitate which does not redissolve in water. The final concentrate (c. 1/100 volume) can be dialysed without loss of activity.

The above procedure involves high salt concentrations causing slow crystallization of salts from ethanol-water mixtures and viscous solutions from which it is difficult to centrifuge or filter insoluble material. It was found much easier to use ethanol precipitation without prior concentration in three stages giving volume diminutions of about 1/10, 1/3, 1/3.

Two volumes of ethanol were added to the crude culture filtrate and it was allowed to stand overnight. The supernatant was siphoned off and the loose precipitate centrifuged to give a compact pellet. A certain amount of mycelial debris was useful in obtaining a coherent precipitate; if the culture filtrate was completely cleared by centrifuging, there was a much lower yield of enzyme. Since the enzyme is not precipitated by ethanol below a certain salt concentration, the aqueous solution was made up to 1 per cent. (w/v) NaCl before the addition of ethanol in the second and third steps.

From one-half to two-thirds of the activity was recovered by this process, but the ease of manipulation far outweighed the increased losses.

(b) Properties of the Concentrate

The β -glucosidase activity of the concentrate was not stable to lyophilization, about one-half the activity being lost. A second lyophilization halved the residual activity and this could be repeated indefinitely. The lyophilized preparation was a fluffy, white powder, in a yield of 1.2 g/l. The ratio of carbohydrate to protein was from 10 : 1 to 20 : 1 in different preparations. An essentially similar product could be obtained from culture filtrates devoid of enzymic activities.

Hydrolysis of the concentrate with boiling 0.5N H_2SO_4 for 2 hr followed by paper chromatography showed the presence of galactose, glucose, and mannose in the estimated ratios 3 : 2 : 1 together with a little uronic acid. Amino sugars were shown to be absent (Thomas (1956) details the tests employed). Galactose was used as the standard sugar in estimating carbohydrate content at the later stages of purification. Hydrolysis with 6N HCl at 100°C for 12 hr followed by paper chromatography gave a chromatogram that could not be readily interpreted owing to humin formation and interference from sugars, but revealed the presence of ninhydrin-reacting spots. In the absence of any evidence for the existence of other ninhydrin-positive substances these have been assumed to be distorted amino acid spots, and the nitrogen of dialysed enzyme preparations has been assumed to be protein nitrogen.

Batch B (Table 1), which was concentrated by evaporation before ethanol precipitation, retained 88 per cent. of the β -glucosidase activity in the dialysed concentrate, but 40 per cent. of the amylase, 65 per cent. of the cellobiase, and 99 per cent. of the sucrase had been eliminated. The β -glucosidase was stable to ethanol and methanol up to 30°C, but sucrase was rapidly destroyed even at 0°C. Use of the higher alcohols to precipitate the β -glucosidase led to inactivation of the enzyme at all temperatures; this enzyme was stable to acetone at low temperatures and salt concentrations, but was rapidly destroyed at high salt concentrations.

The enzyme could not be precipitated by ethanol at low ionic strength nor by saturation with ammonium sulphate. It remained completely in the water phase whatever the pH of an ammonium sulphate-water-ethanol mixture, the only two-phase system in which it was stable. It was not precipitated by specific protein precipitants, even such a general one as the zinc-mercury reagent of Schmid (1953) being ineffective. The only precipitants found were

basic lead acetate and uranyl salts. The only effective adsorbents were activated charcoal and light magnesium carbonate, but no method for eluting the enzyme from charcoal could be discovered.

After precipitation from lead solutions all enzyme activities but the β -glucosidase were eliminated from the most active fraction. Adsorption on magnesium carbonate, as shown in Figure 5, did not lead to differential separation of sucrase (the most persistent enzymic contaminant) from β -glucosidase. Fractionation by lead precipitation was therefore used as the next step in the purification procedure.

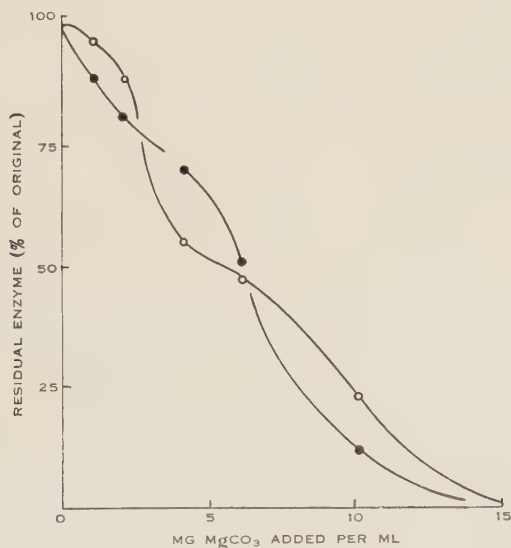


Fig. 5.—Adsorption of enzymes from a dialysed concentrate of *S. atra* culture filtrate on light MgCO_3 . \circ Sucrase. \bullet *p*-Nitrophenyl- β -glucosidase.

(c) Fractionation by Lead Precipitation

When the dialysed concentrate was fractionated by the addition of successive quantities of basic lead acetate solution and centrifuging off the precipitate after each addition, there was no significant increase in the purity of the most active fractions (assessed on the units of enzyme per mg N) over the original material. If the lead was added as the normal acetate to a concentration of 0.1M, no precipitation took place in general, and a series of fractions could be obtained by successive small additions of sodium hydroxide. The initial pH for precipitation varied from experiment to experiment; in one case it was as low as 4.0 and acetic acid had to be added to the normal lead acetate solution. The most active fractions were not the same in different experiments, but in general the bulk of the enzyme activity was precipitated by amounts of NaOH solution sufficient to convert 5-15 per cent. of the normal lead acetate to the basic compound (pH range 5-6).

The most effective reagent for redissolving the lead-precipitated enzyme was found to be a 5 per cent. (w/v) solution of glycine. Any solid matter in the final solution was removed and the latter was then dialysed in a rotary dialyser (16 hr against running tap water). In general all the lead was removed by this dialysis, but occasionally some of it was retained tenaciously. The cause of this anomalous behaviour has not been investigated since the retained lead is removed during the next stage of purification.

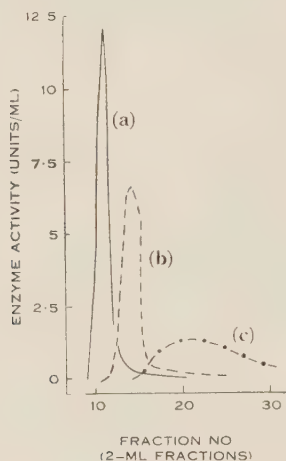


Fig. 6

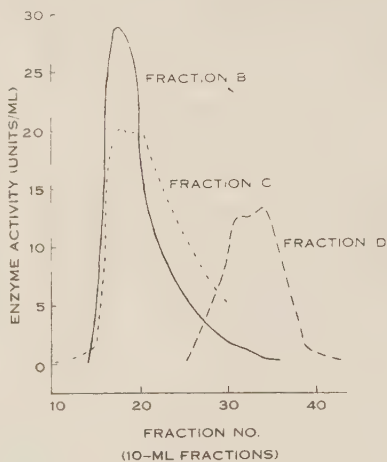


Fig. 7

Fig. 6.—Variation in the elution of 250 units of *S. atra* β -glucosidase from a light MgCO_3 -cellulose column (5 g of 1:1) with concentration of the $(\text{NH}_4)_2\text{SO}_4$ eluting solution. (a) 4 per cent. $(\text{NH}_4)_2\text{SO}_4$; (b) 3.5 per cent. $(\text{NH}_4)_2\text{SO}_4$; (c) 3 per cent. $(\text{NH}_4)_2\text{SO}_4$. Same sample of lead-fractionated enzyme used throughout.

Fig. 7.—Elution of lead-precipitated fractions B, C, D of batch 8 from a light MgCO_3 -cellulose column (40 g of 1:1) with 2.7 per cent. Na_2SO_4 . Curve D is magnified to five times its true height.

The dialysed lead-precipitated enzyme is stable for long periods in the refrigerator. No amylase or cellobiase activity remains, but a little sucrase has occasionally been detected in the least active fractions, which were discarded at this stage.

(d) Chromatography on Magnesium Carbonate Columns

It could be shown (Table 2) that the enzyme in the dialysed solutions from the lead fractionation was reversibly eluted by dilute ammonium sulphate solutions after adsorption on light magnesium carbonate (B.D.H. laboratory reagent). The necessary condition for the use of magnesium carbonate as the adsorbing agent in a chromatographic column was thus fulfilled. The column packing used was equal parts of light magnesium carbonate and Whatman's standard chromatographic cellulose powder, the latter being added merely to

improve flow through the column. Figure 6 shows results obtained by the elution of the β -glucosidase from cellulose-magnesium carbonate columns with

TABLE 2

REVERSIBLE ADSORPTION OF *S. ATRA* β -GLUCOSIDASE ON MAGNESIUM CARBONATE

All adsorptions carried out on 50 mg of light magnesium carbonate from 5 ml of enzyme solution. All apparent activities corrected for the presence of ammonium sulphate

(a) Effect of Enzyme Concentration on Adsorption

Enzyme Concentration (units/ml)		Difference (units/ml)	Units of Enzyme Adsorbed
Before Adsorption	After Adsorption		
1.820	1.360	0.460	2.30
0.920	0.436	0.484	2.42
0.455	0.036	0.419	2.10
0.239	0.016	0.223	1.12
0.117	0.006	0.113	0.57

(b) Effect of Ammonium Sulphate Concentration on Desorption

Desorption Process	Enzyme Concentration (units/ml)	Volume (ml)	Total Units
Enzyme adsorbed	0.298	5	1.49
Enzyme desorbed by 2 per cent. ammonium sulphate	0.097	10	0.97
Enzyme desorbed by 5 per cent. ammonium sulphate	0.143	10	1.43
Enzyme desorbed by 10 per cent. ammonium sulphate	0.153	10	1.53

(c) Effect of Eluting Volume of 2.5 per cent. Ammonium Sulphate

Elution Process	Enzyme Concentration (units/ml)	Volume (ml)	Total Units
Enzyme adsorbed	0.484	5	2.42
Enzyme desorbed by 2.5 per cent. ammonium sulphate	0.493	2	0.98
	0.318	5	1.59
	0.190	10	1.90
	0.152	15	2.28

ammonium sulphate solutions of varying strengths. Similar results were obtained with other enzyme samples, but usually at slightly lower concentrations of the salt (2.5 per cent. ammonium sulphate was the usual optimum);

some samples could be eluted satisfactorily with as little as 1.5 per cent. ammonium sulphate.

Since the ammonia could only be removed from the enzyme fractions by dialysis, which is very tedious when a large number of samples have to be handled, all quantitative studies were carried out by using sodium sulphate as the eluant. The eluting effect of the two sulphates was identical at identical molarities. The results of various experiments using elution with sodium sulphate solutions are set out in Figures 7, 8, and 9. When the salt concentration of the eluant is reduced far enough, there is almost complete separation of the peak of enzyme activity from that of carbohydrate. The enzyme is therefore not firmly associated with most of the carbohydrate present. None the less it was found that the enzyme became progressively less stable as less carbohydrate was associated with it. The enzyme could be recovered quantitatively from

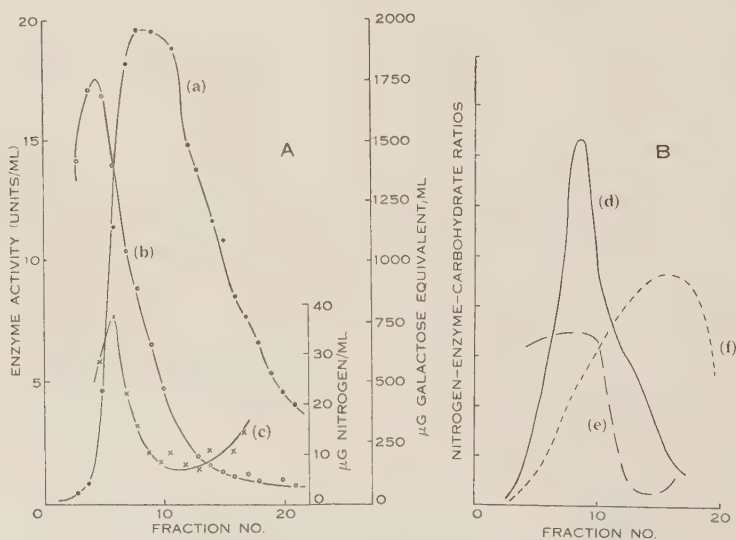


Fig. 8.—A, Analysis of the column fractions from the chromatography of lead-precipitated fraction B of batch 8 (see Fig. 7). (a) Enzyme activity. (b) Carbohydrate. (c) Nitrogen. B, (d) Ratio of enzyme activity to nitrogen. (e) Ratio of carbohydrate to nitrogen. (f) Ratio of enzyme activity to carbohydrate.

adsorption on magnesium carbonate in test tube experiments and 75-80 per cent. could be recovered from columns on elution with strong ammonium sulphate solutions so that the carbohydrate and enzyme peaks were simultaneous. As the enzyme peak lagged behind the carbohydrate peak, progressively less and less enzyme was recovered although nitrogen and carbohydrate were almost quantitatively eluted. Finally, under conditions where little or no carbohydrate was associated with the enzyme (e.g. fractions after the 30th in Fig. 9), very little active enzyme reached the bottom of the column and this little rapidly disappeared on keeping. The later fractions in Figure 9 were inactive after a further 24 hr in the refrigerator. An exceptional sample from lead fractiona-

tion which had a carbohydrate-protein ratio of only 0.3, although perfectly stable in solution at refrigerator temperatures, lost all activity on chromatographing although carbohydrate and nitrogen emerged normally from the column.

It would appear that some at least of the associated carbohydrate is essential for the stability of the enzyme. Helferich and Pigman (1939) have shown that 3.4 per cent. of carbohydrate is tenaciously retained by almond β -glucosidase although there is no direct correlation between activity and carbohydrate content. Helferich, Richter, and Grünler (1937) showed this carbohydrate to be built up from mannose, arabinose, and possibly glucose.

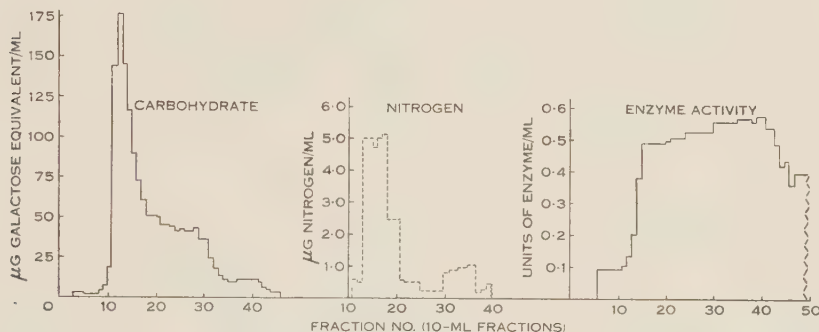


Fig. 9.—Elution of 2000 units of lead-fractionated β -glucosidase from a light MgCO_3 -cellulose column (25 g of 1 : 1) by 1.5 per cent. Na_2SO_4 . The enzyme has been considerably inactivated (see text).

(e) Purification of a Typical Batch of Enzyme

In Table 3 is set out the flow sheet for the purification of a typical batch of enzyme. Table 4 gives the basic data for the estimation of the purity of this enzyme at various stages in the process. Figures 7 and 8 have been chosen both to illustrate the stages in the purification of this batch and the general chromatographic behaviour of the enzyme.

VII. PHYSICOCHEMICAL PROPERTIES OF THE ENZYME

Not more than a few milligrams in dilute solution of highly purified *S. atra* β -glucosidase have yet been prepared. Since this is not sufficient for detailed examination and the behaviour of less pure preparations is dominated by the carbohydrate/protein ratio, no attempt has been made to establish any absolute physicochemical properties. Some incidental observations appear, however, to be of interest in showing how this association modifies the observed properties.

(a) Filter Paper Electrophoresis and Chromatography

When the crude culture filtrate was used the enzyme travelled as a single fairly well-defined spot and the isoelectric point (inaccessible because of inactivation of the enzyme) appeared to be below pH 3. The enzyme in the ethanol-precipitated concentrate was heavily adsorbed at lower pH, but still

TABLE 3
FLOW SHEET FOR PURIFICATION OF THE β -GLUCOSIDASE FROM BATCH 8 OF *S. ATRA* CULTURE FILTRATES

Crude culture filtrate (8180 ml, 4.4 units/ml; 36,000 units)					
Two vols. ethanol added, stood overnight			Ppt. resuspended in water, centrifuged to remove insol. matter (775 ml, 27 units/ml; 20,920 units)		
Supernatant discarded.			Ethanol pptn. and redissoln. repeated twice		
Supernatants discarded.					
Concentrate (50.5 ml, 285 units/ml; 14,400 units) dialysed 16 hr in rotary dialyser against tap water, and centrifuged (68 ml, 218 units/ml; 14,800 units)					
Made 0.1M in $\text{Pb}(\text{CH}_3\text{COO})_2$ by addition of 1/9 vol. of $\text{M Pb}(\text{CH}_3\text{COO})_2$					
N NaOH (0.5 ml) added, equilibrated by shaking, ppt. centrifuged off	N NaOH (0.5 ml) added to supernatant (1.0 ml total) ppt. centrifuged off	Further 0.5 ml N NaOH added to supernatant (1.5 ml total), ppt. centrifuged off	Further 0.5 ml N NaOH added to supernatant (2.0 ml total) ppt. centrifuged off	Further 1.0 ml N NaOH added to supernatant (3.0 ml total) ppt. centrifuged off	Further 2.0 ml N NaOH added to supernatant (5.0 ml total) ppt. centrifuged off
Fraction A (24.8 ml, 17 units/ml; 420 units) discarded.	Fraction B (27.0 ml, 233 units/ml; 6290 units)	Fraction C (26.0 ml, 198 units/ml; 5140 units)	Fraction D (26.5 ml, 48 units/ml; 1270 units)	Fraction E (no activity) discarded.	Fraction F (no activity) discarded.
Ppt. washed with water, dissolved in 25ml 5% glycine, insol. matter centrifuged off					
Dialysed 16 hr in rotary dialyser against tap water, centrifuged					
(31.2 ml, 214 units/ml; 6680 units)					
Adsorbed on column of light MgCO_3 -cellulose powder (25 g of 1 : 1), washed with water, eluted with 2.5% $(\text{NH}_4)_2\text{SO}_4$ (see Fig. 6 for results)					
Most active fractions combined and dialysed.					
Most active fractions combined and dialysed.					

moved as a well-defined spot at pH 8-10; extrapolation resulted in a similar value for the isoelectric point. The lead-precipitated enzyme and, still more, column fractions with carbohydrate/protein ratios of 2-3 were so heavily adsorbed on the paper, no matter how it had been treated, that no deductions were possible.

Similar behaviour was observed with paper chromatography, the crude enzyme moving freely in methanol-buffer mixture and semi-purified preparations being tightly held at the point of application.

TABLE 4
ANALYTICAL DATA FOR BATCH 8 AT VARIOUS STAGES OF PURIFICATION

Stage	Units of Enzyme Activity per mg N	Units of Enzyme Activity per mg Galactose Equivalent	Polysaccharide/ Protein Ratio	Purification Factor
Ethanol-precipitated and dialysed	525	5.95	12.7	1
Lead-precipitated:				
Fraction B	2280	25.7	12.8	4.3
Fraction C	1580	23.1	9.9	3.0
Fraction D	770	18.6	6.0	1.45
Column fractions:				
B 21-22	2660	66	5.8	5.1
B 23-26	2410	100	3.5	4.6
C 17-21	2280	132	2.5	4.3

(b) Adsorption

In keeping with the increased adsorption on filter paper of small amounts of enzyme during its progressive purification, the same behaviour was observed with a variety of other adsorbents. The adsorbability of the enzyme on magnesium carbonate rises rapidly as carbohydrate is removed from enzyme preparations and much smaller columns can be used than would be predicted from experiments on the adsorption of the crude enzyme. A slight adsorption on calcium phosphate gel and calcium carbonate is sometimes evident.

It was not possible to find conditions in which the enzyme was retained by columns of ion-exchange resins. The activity disappeared on contact with strongly acidic cation exchangers, alone or in mixed-bed resins, but this could be shown to be due to the normal acid inactivation of the enzyme.

(c) Optical Properties

Dilute solutions of the enzyme showed no absorption in the visible range after lead fractionation (carbohydrate/protein ratio about 12:1), but an intense end-adsorption in the ultraviolet. Only when the carbohydrate/protein

ratio fell to about 2:1 after passage through a column was the characteristic maximum at 2800 Å in the protein ultraviolet adsorption curve visible as a hump on the enzyme absorption curve. Figure 10 illustrates this point.

The nature of the material responsible for this intense ultraviolet absorption is not known. The sum of total carbohydrate estimated by the anthrone method and protein ($6.25 \times N$) usually came to about 90-95 per cent. of the dry weight. Analytical figures were a little high in carbon and hydrogen and much too high in oxygen for a mixture of carbohydrate and protein, and a component with the type of analytical figures expected for an oxygenated organic acid is suggested.

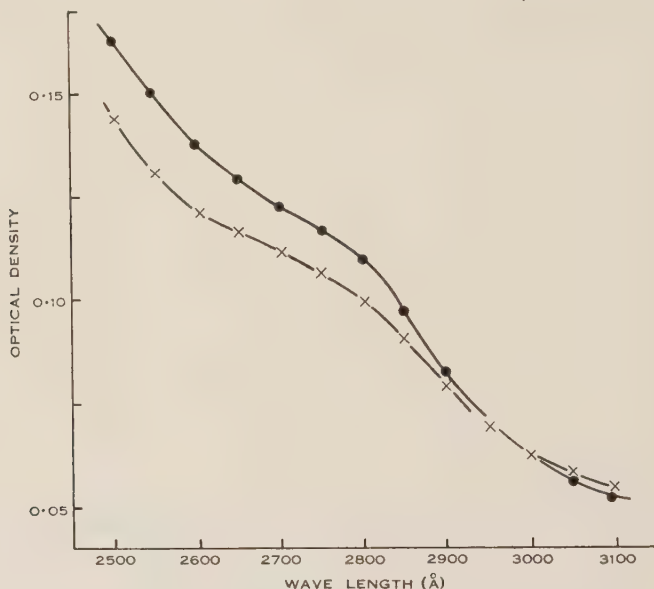


Fig. 10.—Ultraviolet absorption spectrum of two samples of *S. atra* β -glucosidase eluted from a $MgCO_3$ column. ● Carbohydrate/protein ratio 2.5. × Carbohydrate/protein ratio 3.5.

(d) Electrophoresis

A sample of dialysed, lead-fractionated enzyme, free of ash and with an enzyme activity of about 2000 units per mg N and a carbohydrate/protein ratio of 12:1 was submitted to electrophoresis in the Tiselius apparatus by Mr. J. M. Gillespie. The electrophoresis pattern showed rapid flattening of the boundary with re-sharpening on reversal of the current. This behaviour is consistent with the existence of a considerable number of molecular species, of not very dissimilar mobilities.

VIII. DISCUSSION

Thomas (1956) found that his cellulase preparations from *S. atra* contained a polysaccharide very similar in properties to that encountered in the β -glucosi-

dase preparations. These polysaccharides seem to be a characteristic product of *S. atra*, but in the case of the cellulase preparations they were not present in such a large excess as to dominate the behaviour of the enzyme. The dependence of the stability of the *p*-nitrophenyl- β -glucosidase on the presence of an associated but separable polysaccharide resembles the experience of Fischer (1954) with yeast invertase.

Although the variation in the physicochemical properties of the enzyme with the amount of associated polysaccharide is such as to make its complete purification beyond the scope of the present study, it will be shown in Parts V and VI of this series that this variation is not incompatible with constancy of enzymic properties. The constant value of K_m against various substrates and the reproducibility of the peculiar features of the pH-activity curve make it probable that a single enzymic species is being studied. This is supported by the single peak of enzyme activity obtained on elution from magnesium carbonate columns.

By correlating the present observations with those of Thomas (1956) (Part VII of this series) it can be shown that *S. atra* produces at least the following four enzymes concerned in the hydrolysis of β -glucosidic linkages:

- (i) The β -glucosidase here studied; incapable of splitting cellobiose.
- (ii) A cellobiase, probably capable also of splitting aryl- β -glucosides. The upper limiting size of substrate for this enzyme is not known but the enzyme producing reducing groups from carboxymethylcellulose studied by Jermyn (1953a) and cellobiase activity always seem to occur together.
- (iii) The adaptive "viscometric cellulase" of Thomas (1956). This enzyme attacks soluble cellulose derivatives with cellotriose as the smallest molecule attacked; it has no action on cellobiose. Preparations containing this activity also attack many insoluble celluloses (the inseparability of the two activities has not yet been rigorously proved) but not unscoured cotton duck. The ability of the fungus to grow on this substrate may be provisionally attributed to the presence of (iv).
- (iv) A "swelling factor." This enzyme opens up highly crystalline celluloses to attack by the cellulase (Thomas 1955).

The full biological significance of the observations recorded in Parts IV, V, and VI of this series cannot be discussed until the mode of action of yet unstudied enzymes has been elucidated and the physiology of their production understood.

IX. ACKNOWLEDGMENTS

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FUNGAL CELLULASES

V. ENZYMIC PROPERTIES OF *STACHYBOTRYS ATRA* β -GLUCOSIDASE

By M. A. JERMYN*

[Manuscript received April 14, 1955]

Summary

The effect of enzyme and substrate concentration, pH, temperature, and the nature and concentration of added salts on the activity of the β -glucosidase from *Stachybotrys atra* have been investigated. The inactivation of the enzyme by heat and acid has also been studied.

Energies of activation and Michaelis constants have been calculated for the enzyme under various conditions.

I. INTRODUCTION

Part IV of this series (Jermyn 1955) described the isolation and partial purification of a β -glucosidase from culture filtrates of *Stachybotrys atra*. The present paper summarizes some of the data necessary for the characterization of this enzyme. Only such information on substrate specificity as is necessary to correlate the other data will be considered here; Part VI in this series will consider the relation of the enzyme to its substrates and competitive inhibitors.

The most recent review devoted to β -glucosidases is that of Veibel (1950) which summarizes and gives references to much of the older literature.

II. METHODS

The methods for estimating β -glucosidase activity, outlined in Part IV of this series (Jermyn 1955), have been modified as required to estimate the effect of different variables. Thus in the *p*-nitrophenol- β -glucosidase technique the volume was held constant at 5 ml while pH and buffer, enzyme, substrate, and salt concentrations were varied by suitable modification of the original solutions. The 2 ml of 7.5 per cent. (w/v) K_2HPO_4 solution added at the end of the chosen period of reaction was occasionally modified to 75 per cent. (w/v) K_2HPO_4 for strongly acid reaction media or even to 10 per cent. (w/v) K_2CO_3 in order to bring the final pH to *c.* 8.5. In some instances insoluble precipitates had to be centrifuged off before making readings in the spectrophotometer. So long as the final pH was in the indicated range, the concentration of salts had no significant effect on the optical density.

Both techniques could be scaled up by any suitable factor so that samples of 5 ml (*p*-nitrophenol method) or 10 ml (Folin-Ciocalteu method) could be removed at intervals over a period of time. Temperatures were controlled throughout by the use of a thermostat bath which could be regulated to $\pm 0.1^\circ C$.

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The enzyme preparations used were lead-precipitated and dialysed fractions prepared by the methods outlined in Part IV. Only material with activity greater than 2000 units per mg N was used; the polysaccharide/protein ratio ranged from 10 to 15. Variations in the amount of polysaccharide present did not seem to have any detectable effect on enzymic properties. The Michaelis constant against *p*-nitrophenyl- β -glucoside (the property which on the available evidence should be most affected by carbohydrate impurities) was tested for

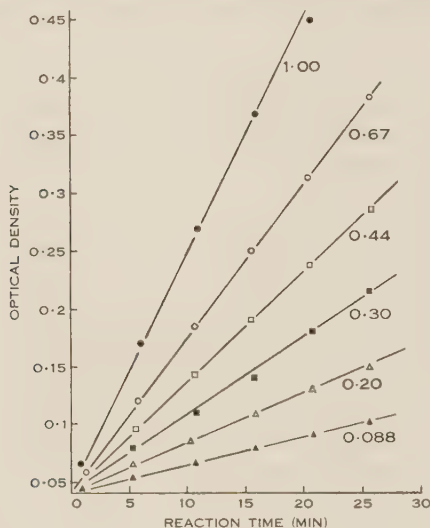


Fig. 1

Fig. 1.—Optical density at 400 $m\mu$ of standard enzyme reaction mixture at various times after adding enzyme. 5-ml samples withdrawn and added to 2 ml of 7.5 per cent. K_2HPO_4 solution. Incubation temperature 28°C. The numbers are relative enzyme strengths on an arbitrary scale.

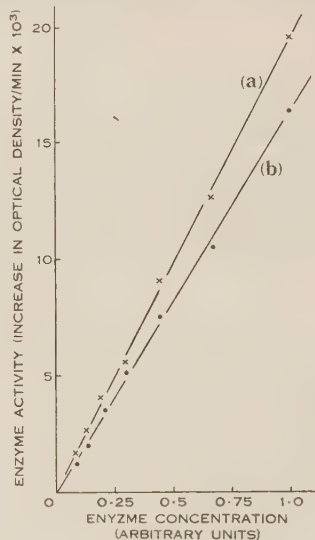


Fig. 2

Fig. 2.—Effect of enzyme concentration on rate of decomposition of *p*-nitrophenyl- β -glucoside. Reaction velocities are calculated from the slope of lines similar to those of Figure 1. Incubation temperature 28°C. Under otherwise standard conditions. (a) Substrate concentration $3 \times 10^{-3}M$. (b) Substrate concentration $3 \times 10^{-4}M$.

each sample and found to be sensibly constant. Nor was the Michaelis constant affected by the fall of the polysaccharide/protein ratio to 2 in stable column fractions.

III. ENZYME KINETICS

(a) Effect of Time and Enzyme Concentrations

Under conditions where the β -glucosidase was stable (in the pH range 4-10 and at temperatures below 45°C) the rate of hydrolysis was found to be linear with time in the presence of excess substrate. When the slope of these lines was plotted against enzyme concentration, another straight line resulted (see Figs. 1 and 2).

The demonstration of first order kinetics with regard to enzyme concentration is a justification of the method described in Part IV of this series for enzyme estimation, since the standard concentration of *p*-nitrophenyl- β -glucoside (10^{-3}M) is well above K_m for this substrate.

TABLE 1

EFFECT OF pH AND BUFFER STRENGTH ON THE MICHAELIS CONSTANT FOR THE HYDROLYSIS OF *p*-NITROPHENYL- β -GLUCOSIDE BY THE β -GLUCOSIDASE OF *S. ATRA*

Buffer	Michaelis Constant ($\times 10^5$)			
	pH 4	pH 5	pH 6	pH 7
Sodium phosphate-citric acid*	3.4	2.9	6.8	1.7
Sodium phosphate-citric acid†		4.4	3.8	3.0
Sodium acetate, 0.2M	2.4	4.9		
Sodium acetate, 0.1M	2.5	2.7		

* Four times McIlvaine concentration.

† One-tenth McIlvaine concentration.

(b) Course of Total Hydrolysis

When the course of hydrolysis of the *p*-nitrophenyl- β -glucoside is followed by withdrawing samples over a period of time, the curve relating percentage substrate decomposed to incubation time falls considerably below the asymptotic

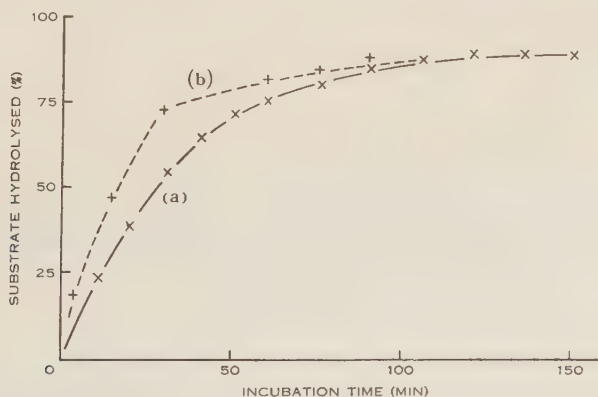


Fig. 3.—Time course of the hydrolysis of *p*-nitrophenyl- β -glucoside by the *S. atra* β -glucosidase under standard conditions. (a) Substrate concentration $3 \times 10^{-5}\text{M}$.
(b) Substrate concentration $5 \times 10^{-5}\text{M}$.

approach to 100 per cent. decomposition expected from normal first order kinetics (Fig. 3). This behaviour led to the observation that the glucose liber-

ated by hydrolysis is a powerful competitive inhibitor of the enzyme. No phenol tested was found to exert a comparable effect.

(c) Effect of pH

In Figure 4 are set out pH-activity curves for the *S. atra* β -glucosidase against a number of substrates. McIlvaine (citric acid-sodium phosphate) buffers were used throughout, since they covered the whole range of enzyme activity. Almond emulsin measured under exactly identical conditions (Fig. 5) gives a single symmetrical peak and the secondary peak given by the *S. atra* enzyme at around pH 6 appears to be characteristic and not due to interactions

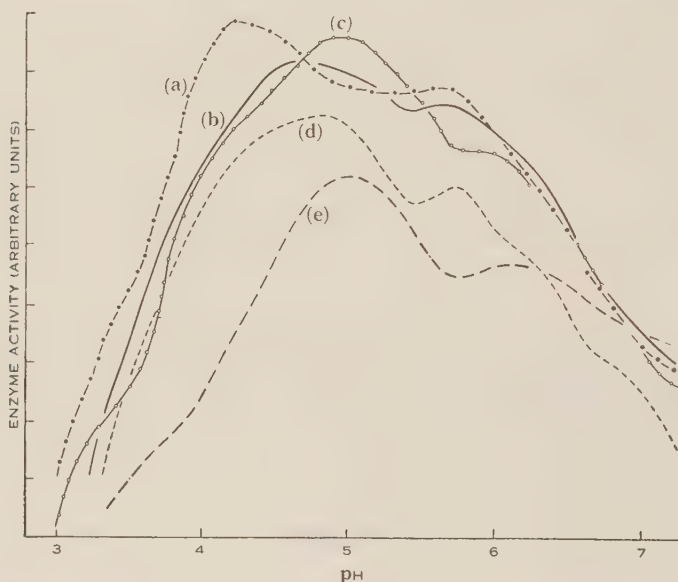


Fig. 4.—pH-activity curves for the *S. atra* β -glucosidase against a number of β -glucosides. Citrate-phosphate buffers at 28°C under identical conditions (10^{-3} M substrates). (a) 2-Naphthyl- β -glucoside. (b) Phenyl- β -glucoside. (c) *m*-Cresyl- β -glucoside. (d) *p*-Chlorophenyl- β -glucoside. (e) *o*-Hydroxymethylphenyl- β -glucoside (salicin).

which take place between the buffering salts and β -glucosidases in general.

Since it was later found that enzyme activity was affected by the nature of the buffering ion and salt concentration, and that K_m varied with pH and buffer, an attempt was made to construct a pH-activity curve in which these disturbing factors were eliminated.

The points of the curves in Figure 6 were obtained by a process of double extrapolation. A single stock solution of suitably diluted enzyme (shown to be stable over the course of the experiments) was employed throughout. Using this enzyme solution and a range of substrate concentrations where the

Michaelis-Menten relationship was known to hold, V_{\max} at infinite substrate concentration was deduced for any given pH and buffer concentration by the extrapolation of Lineweaver-Burk plots. These calculated values of V_{\max} were then plotted against buffer concentration and the curve extrapolated (no way was found of obtaining a linear plot) to give a value for V_{\max} at zero buffer concentration.

The curves of Figure 6 show that the secondary peak in the curve for citrate-phosphate buffers has been substantially reduced by this procedure, and that the curve now has a single flat maximum and a slight shoulder. The non-equivalence of the citrate-phosphate and acetate curves shows that specific ion effects have not been eliminated.

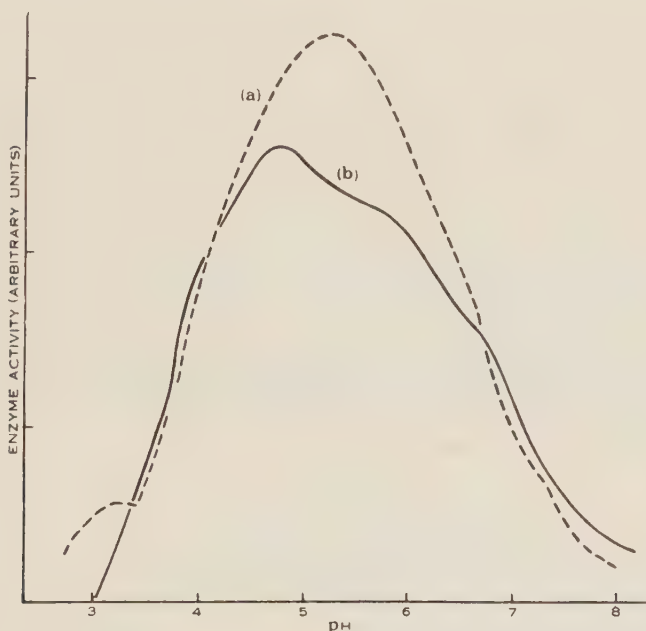


Fig. 5.—Comparison under identical conditions of the pH-activity curves of (b) *S. atra* β -glucosidase and (a) almond emulsin against *p*-nitrophenyl- β -glucoside. Citrate-phosphate buffers at 28°C.

(d) Effect of Substrate Concentration

Figure 7 illustrates the relationship between substrate concentration and rate of hydrolysis for two substrates. At high substrate concentrations the results do not accord with the Michaelis-Menten equation. This is one aspect of the general inhibition by excess of polyhydroxy compounds which will be discussed in Part VI of this series. However, at low substrate concentrations, the experimental results lead to rectilinear plots according to the method of Lineweaver and Burk (1934) and K_m can be calculated.

Figure 8 sets out Lineweaver-Burk plots for a number of substrates. For *p*-nitrophenyl- β -glucoside it has been found that this apparent K_m under the

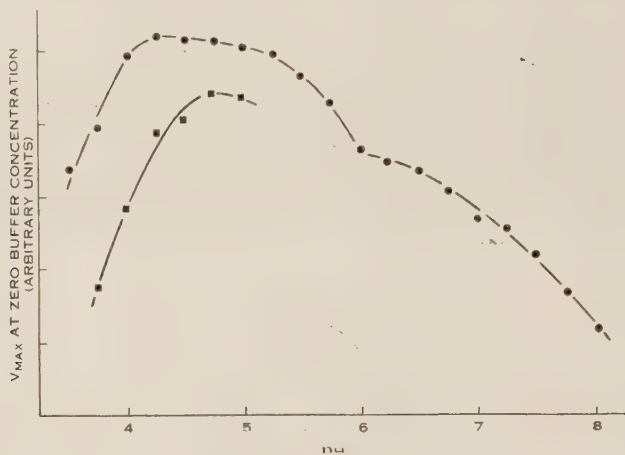


Fig. 6.—Enzyme activity of *S. atra* β -glucosidase extrapolated to zero buffer concentration and infinite substrate concentration (against *p*-nitrophenyl- β -glucoside at 28°C). ● Citrate-phosphate buffers. ■ Sodium acetate buffers. The same enzyme solution was used to determine both curves.

standard conditions (28°C and pH 5 McIlvaine buffer), is not affected by enzyme concentration or the use of different samples of enzyme.

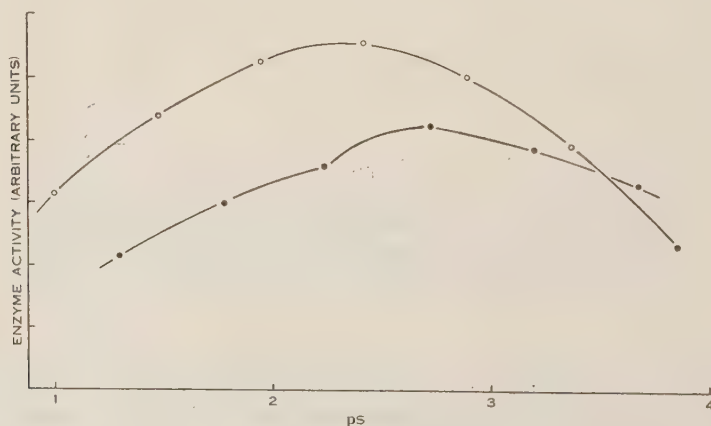


Fig. 7.—Effect of substrate concentration on the activity of *S. atra* β -glucosidase under standard conditions. ○ Phenyl- β -glucoside. ● *p*-Nitrophenyl- β -glucoside.

The values of V_{\max} calculated from data obtained at substrate concentrations below $10^{-3}M$ although fictitious, are extremely useful in comparing the

activity of the enzyme against various substrates with the disturbing effects of differing K_m values eliminated.

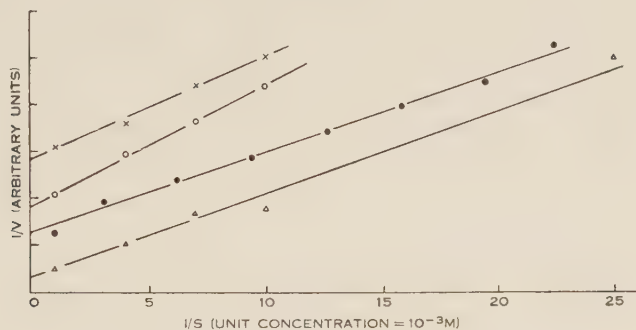


Fig. 8.—Lineweaver-Burk plots of the effect of low substrate concentrations on the velocity of hydrolysis of various β -glucosides by *S. atra* β -glucosidase. Standard conditions, 28°C. \times 2-Naphthyl- β -glucoside. \circ Phenyl- β -glucoside. \bullet *p*-Nitro-phenyl- β -glucoside. \triangle *p*-Cresyl- β -glucoside.

(e) Effect of pH, Buffer, and Salt Concentration on the Michaelis Constant

In Table 1 are set out changes in the K_m values obtained for *p*-nitrophenyl- β -glucoside using acetate and citrate-phosphate buffers at different pH and two different levels of concentration; linear Lineweaver-Burk plots were obtained under all the conditions used. The differences obtained are significant (K_m at

TABLE 2

EFFECT OF 0.01M BUFFERS ON THE ACTIVITY OF *S. ATRA* β -GLUCOSIDASE AGAINST 10^{-5} M *p*-NITROPHENYL- β -GLUCOSIDE AT 28°C

The same dialysed enzyme sample was used throughout

Buffer (pH 5)	Enzyme Activity (relative to acetate buffer)
Sodium phosphate	0.85
Sodium succinate	0.71
Sodium citrate	0.94
Sodium phthalate	0.99
Sodium acetate	1.00
Sodium phenylacetate	0.93
Anilinium hydrochloride	1.07
Pyridinium hydrochloride	0.91

pH 5.0 under standard conditions is reproducibly 4.55×10^{-5} M) but not profound. It is impossible on this limited evidence to offer a rational explanation of the observed effects.

(f) *Effect of the Ionic Environment on the Activity of the Enzyme*

The observations which are incorporated in Figure 6 and Table 1 would lead to the expectation that all changes in the ionic environment would profoundly affect the activity of the enzyme. This was found to be the case and any statement of enzyme activity is accordingly meaningless unless this environment is rigidly specified.

Table 2 shows the variation in enzyme activity with variation in the nature of buffering ions at constant pH; and Figure 9 shows the variation with

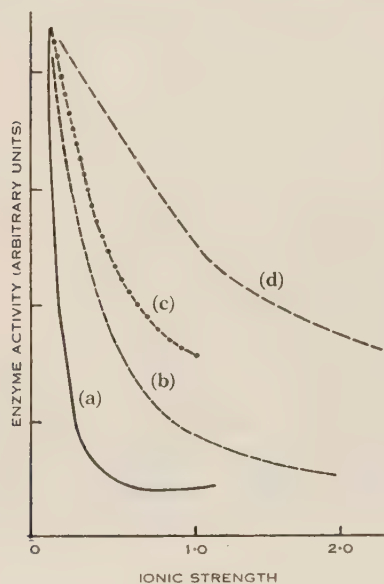


Fig. 9

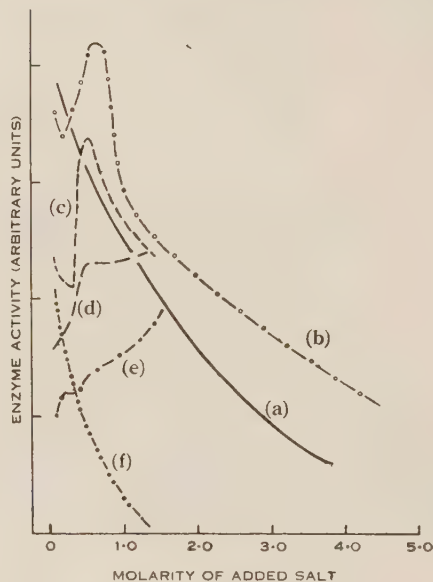


Fig. 10

Fig. 9.—Specific effects of the concentration of various buffering ions on the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase. (a) Pyridinium-HCl, pH 5.0. (b) Sodium acetate, pH 5.0. (c) NaH_2PO_4 . (d) Sodium phosphate-citric acid, pH 5.0. In this and the next figure the buffer strengths given are those finally attained in the incubation mixture.

Fig. 10.—Effect of neutral salts on the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase in the presence of various buffers. All buffers readjusted to pH 5.0 after the addition of the salts. (a) McIlvaine buffer with added NaCl. (b) 0.02M Pyridinium-HCl with added NaCl. (c) 0.02M Sodium acetate with added NaCl. (d) 0.02M Sodium acetate with added Na_2SO_4 . (e) 0.02M Sodium acetate with added MgCl_2 . (f) 0.02M Sodium acetate with added MgSO_4 .

buffering ion concentration. These effects are not limited to buffering ions alone; the influence of neutral salts is shown in Figure 10. It is apparent that various salts influence the activity in a way which cannot be explained by greater or lesser ionic strength. The significance of the secondary peak of activity as the concentration of sodium chloride is increased in dilute buffers is quite uncertain.

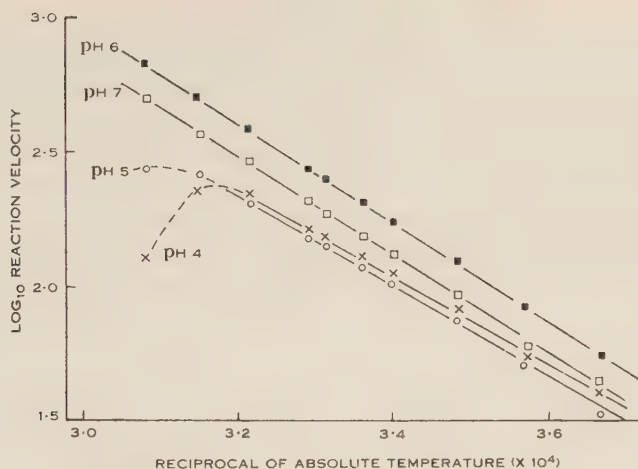


Fig. 11.—Effect of temperature on the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase at four pH's. Reaction time 20 min; substrate concentration 10^{-3} M. Sodium phosphate-citric acid (McIlvaine) buffers.

(g) Energy of Activation

In spite of the profound effect of various alterations of the environment on the activity of the β -glucosidase, it was found that the apparent energy of activation was little altered by changes in pH and substrate. Figure 11 shows

TABLE 3
ENERGY OF ACTIVATION* FOR THE HYDROLYSIS OF VARIOUS β -GLUCOSIDES BY THE β -GLUCOSIDASE OF *S. ATRA* IN SODIUM PHOSPHATE-CITRIC ACID (McILVAINE) BUFFERS

Substrate ($\times 10^{-3}$ M)	pH	Temperature Range ($^{\circ}$ C)	Energy of Activation \pm S.D. (calories/mol.)
<i>p</i> -Nitrophenyl- β -glucoside	7.0	0.50	8390 ± 110
<i>p</i> -Nitrophenyl- β -glucoside	6.0	0.50	8540 ± 140
<i>p</i> -Nitrophenyl- β -glucoside	5.0	0.44	7800 ± 200
<i>p</i> -Nitrophenyl- β -glucoside	4.0	0.38	7440 ± 220
Phenyl- β -glucoside	5.0	0.28	9300 ± 300
<i>o</i> -Methylphenyl- β -glucoside	5.0	0.28	8600 ± 150
<i>o</i> -Hydroxymethyl-phenyl- β -glucoside (salicin)	5.0	0.28	8020 ± 240

* Calculated from the regression of $\log k$ on $1/T_{abs}$.

that the hydrolysis of *p*-nitrophenyl- β -glucoside follows the Arrhenius relation-ship in the pH range 4-7; the departure of the plot of $\log k$ against $1/T_{\text{abs}}$ from a straight line at the lower pH values occurs only at temperatures where the enzyme can be shown to be inactivated. Table 3 shows some derived values for the energy of activation; there is not sufficient evidence to decide whether the apparently significant difference between the energy of activation for the hydrolysis of *p*-nitrophenyl- β -glucoside at pH 4.0 and 6.0 is genuine or due to slight inactivation of the enzyme at the higher temperatures during incubation.

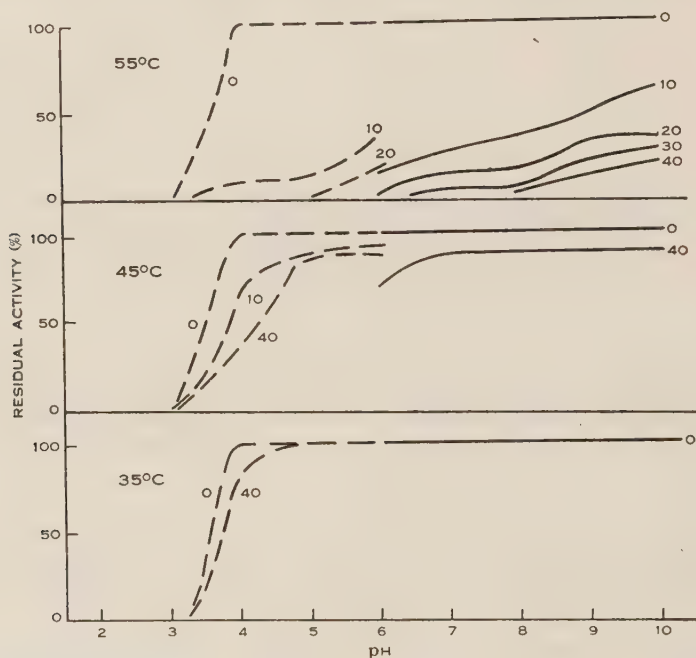


Fig. 12.—Stability of *S. atra* β -glucosidase at different pH's and temperatures. Broken lines, ACA buffers; full lines, ETE buffers. The numbers represent minutes of incubation.

IV. INACTIVATION OF THE ENZYME

(a) Influence of pH and Temperature

A solution of dialysed *S. atra* β -glucosidase (about 4 units per ml) in a buffer was incubated at the required temperature, samples withdrawn from time to time, and the residual activity estimated after dilution with cold McIlvaine buffer (pH 5). In order to cover the maximum range with simple ionic species, the buffers used were 0.05M ethanalamine-triethanolamine hydrochloride adjusted with NaOH (Thies and Kallinich 1953) and 0.05M sodium acetate-chloroacetate (equimolar proportions) adjusted with HCl. These buffers will be referred to as ETE and ACA buffers respectively; besides the

positively or negatively charged buffering species, they contain only varying amounts of sodium and chloride ions (Fig. 12).

It is evident that the resistance of the enzyme to temperature inactivation rises with increasing pH as far as the maximum (pH 10) used in this study and that the enzyme is more stable in ACA than in ETE at pH 6. The change from relative stability to complete inactivation with decreasing pH is very sharp; the enzyme is stable to above 30°C at pH 4 in ACA but inactivated immediately on mixing at pH 3.6, even at 0°C. Small differences in experimental conditions gave apparently inconsistent results and it was impossible to determine the

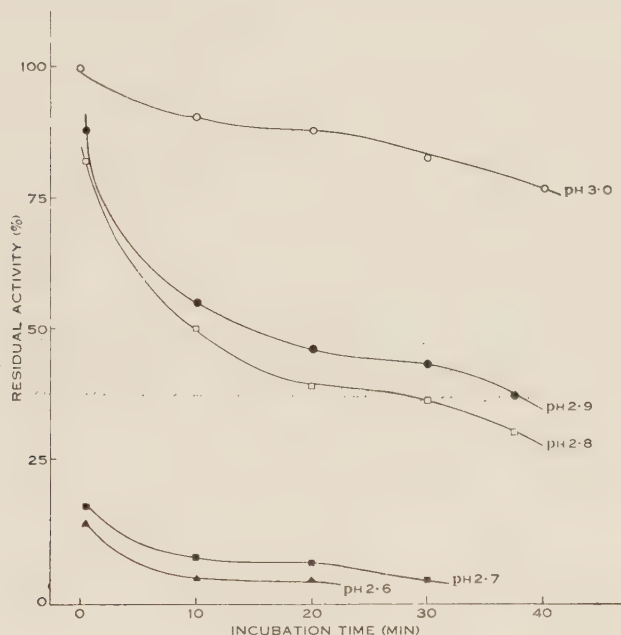


Fig. 13.—Inactivation of *S. atra* β -glucosidase in sodium phosphate-citric acid (Mellvaine) buffers. Enzyme concentration 2 units/ml; incubation temperature 20°C. The pH's at which the inactivation curves were obtained are shown on the diagram.

boundary more accurately than the range 3.6-4.0. In sodium phosphate-citric acid buffers it was possible to demonstrate the transition over a narrow range of pH values (Fig. 13). The enzyme is considerably more resistant to inactivation by acid in phosphate-citrate buffer than in ACA.

The ionic environment thus apparently affects the stability as well as the activity of the enzyme. That these effects extend to neutral salts for the stabilization may be seen from Figure 14. The maximum stabilization by an intermediate concentration of added sodium chloride is perhaps parallel to the similar effect of sodium chloride on the activity of the enzyme in certain buffers (Fig. 10).

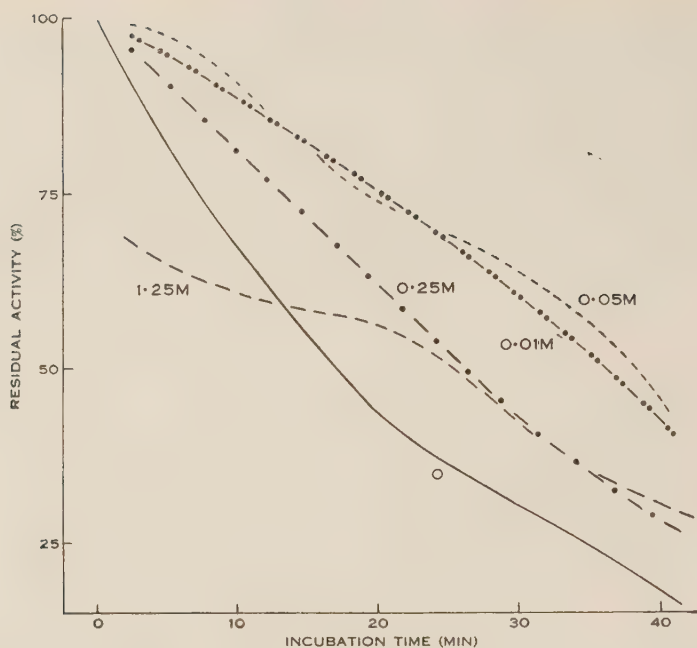


Fig. 14.—Effect of various concentrations of NaCl on the inactivation of *S. atra* β -glucosidase in ACA buffer (0.05M, pH 4.0) at 45°C. The figures give the concentration of added NaCl.

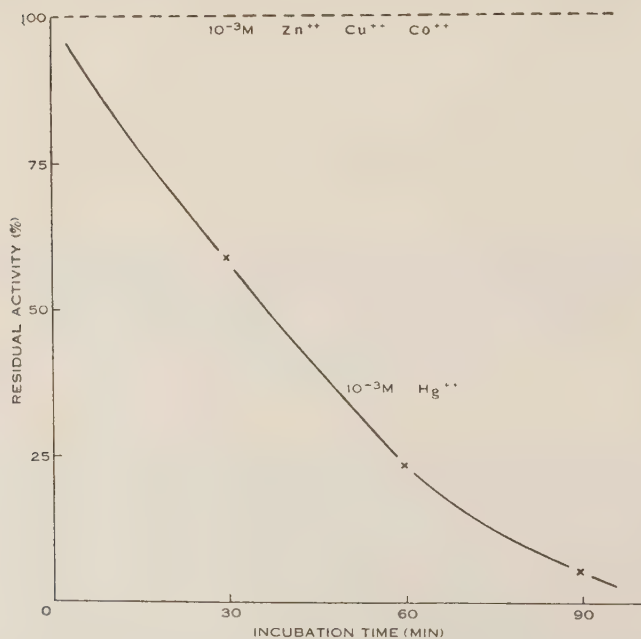


Fig. 15.—Progressive inactivation of *S. atra* β -glucosidase (2 units/ml) in sodium acetate buffer (0.1M, pH 5.0) by 10^{-3}M HgCl_2 at 28°C.

(b) Inactivation by Heavy Metals

A large number of anions and cations were examined to see whether they had any specific effect on the inactivation of the enzyme (in McIlvaine buffer at pH 5.0). The only effective ions were found to be the heavy metal cations mercury, silver, and thallium. This inactivation was irreversible and, since lead has no irreversible effects on the activity (see Part IV of this series), it does not appear to be due to reaction with sulphydryl groups. Thomas (1956) has shown that the cellulase of *S. atra* is inactivated by mercuric salts, but is insensitive to sulphydryl reagents.

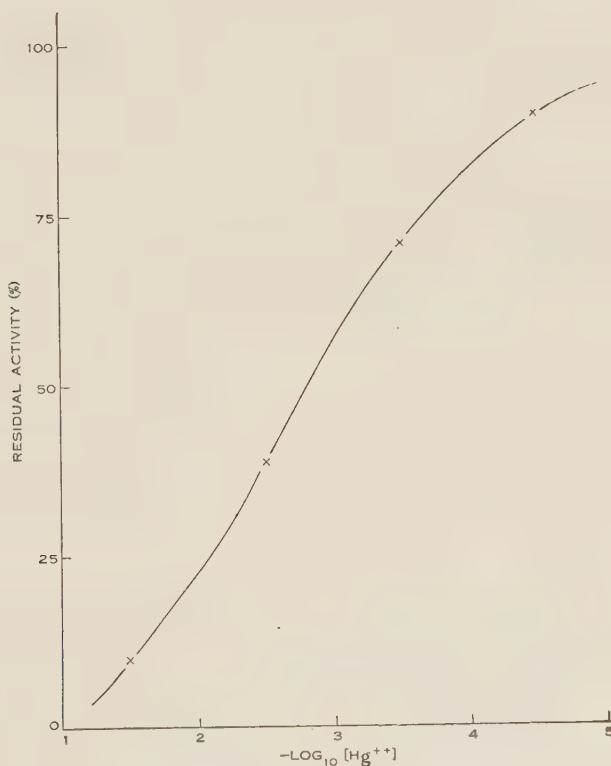


Fig. 16.—Dependence of the rate of inactivation of *S. atra* β -glucosidase on HgCl_2 concentration. Residual activity after 30 min incubation at 28°C ; 2 units/ml of enzyme in sodium acetate buffer (pH 5.0, 0.1M).

Since thallium and silver slowly gave precipitates in the incubation medium used, all further work was done using mercury. The progressive inactivation of the enzyme with time and the dependence of the rate on the concentration of mercuric ion (see Figs. 15 and 16) suggest that the inactivation is due to some relatively slow chemical reaction.

IV. DISCUSSION

Most of the data presented here for the β -glucosidase of *S. atra* are more suggestive of lines for future research than informative in themselves. The main purpose of this study was to determine whether the enzyme possessed unusual properties that would have to be taken into account in any scheme for interrelating the activities of the various β -glucoside-splitting enzymes of *S. atra*. Judged by this criterion, the β -glucosidase here studied is in no way a remarkable enzyme and all the data presented can be paralleled by other workers' results as summarized in Veibel (1950).

V. ACKNOWLEDGMENT

I am indebted to Miss S. Plaisted for carrying out the greater part of the experimental work.

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FUNGAL CELLULASES

VI. SUBSTRATE AND INHIBITOR SPECIFICITY OF THE β -GLUCOSIDASE OF *STACHYBOTRYS ATRA*

By M. A. JERMYN*

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Summary

A large number of compounds with glycosidic linkages have been tested as substrates for the β -glucosidase of *Stachybotrys atra*. The enzyme appears to be specific for β -glucosides and all configurational alterations to the D-glucopyranose ring or substitutions in it lead to non-substrates; phenyl- β -thiogluco-side is a substrate, however. Aryl- β -glucosides have a higher affinity for the enzyme than alkyl- β -glucosides and no hydrolysis of cellobiose by the enzyme can be demonstrated. *ortho*-Substitution in aryl- β -glucosides leads to a marked decrease in the affinity between enzyme and substrate.

All substrates of the enzyme are competitive inhibitors of its hydrolysis of *p*-nitrophenyl- β -glucoside as are also glucose and a number of other sugars and sugar acids. All polyhydroxy compounds tested affect β -glucosidase activity, many raising it slightly at moderately high concentrations and depressing it as the concentration is raised further. These compounds do not appear to act as orthodox non-competitive inhibitors. The combination of the two effects complicates the behaviour of the less efficient competitive inhibitors.

In general, the Michaelis constant for the hydrolysis of substrate is not identical with its inhibitor constant for the competitive inhibition of the hydrolysis of *p*-nitrophenyl- β -glucoside. This result is contrary to the predictions of the Michaelis-Menten theory.

It appears that there must be at least two types of binding centre in the enzyme molecule and the nature of the complex between enzyme, substrate, and third molecular species is discussed.

I. INTRODUCTION

Beta-glucosidase has been the classic subject for studying enzyme specificity, perhaps because of the ease with which a wide variety of substrates for the enzyme can be synthesized. It has been known since the work of Willstätter and Kuhn (1924) that the specificities of β -glucosidases even from closely-related plant species are different.

Many of the enzyme preparations used were not highly purified and even in the case of the much-studied kernel emulsins of *Prunus* species it is doubtful if the behaviour of a single enzyme species has been studied in any case. Thus Miwa and Tanaka (1949) have shown that the β -glucosidase, β -galactosidase, and β -xylosidase of apricot emulsin can be at least partially separated although they are often thought to be activities of a single enzyme. For fungal preparations the separation of glycosidase activities is often somewhat easier, e.g. β -xylosidase from β -glucosidase (Morita 1952), β -glucosidase from β -galactosidase (Niwa 1951) in *Aspergillus niger* preparations, and β -galactosidase from β -glucosidase in taka-diastase (Nishizawa 1942).

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The homogeneity of the β -glucosidase itself has not been shown in many cases and demonstrations have depended on showing the constancy of the ratios of the activity towards various substrates both in different preparations and in the same preparation at different stages of purification. Nishizawa and Wakabayashi (1951) have used a varying ratio of activities in different preparations to show that malt β -glucosidase is probably the sum of at least two enzymes, one a "cellobiase." Jermyn (1952) found that *A. oryzae* preparations contained a number of β -glucosidases and obtained evidence that their specificities were different. Only one of these enzyme components was active against *p*-nitrophenyl- β -glucoside although the others hydrolysed a wide variety of β -glucosides. Similarly Hattori and Kurihara (1950) obtained a β -glucosidase from *Aucuba japonica* ("aucubinase") that specifically attacked the β -glucoside aucubin. The isolation of distinct narrow-specificity α -glucosidases from a yeast strain has been claimed by Hestrin and Lindegren (1950). Levvy and Marsh (1954) have shown that claims for narrow-specificity enzymes must be treated critically, taking as an instance the β -glucuronidase from *Scutellaria baicalensis* (Miwa 1932) where a substrate of high affinity for the enzyme, thus acting as a powerful competitive inhibitor of the hydrolysis of other potential substrates, is a persistent impurity in supposedly purified enzyme preparations.

Parts IV and V of this series (Jermyn 1955*a*, 1955*b*) have described the isolation of a highly purified β -glucosidase from culture filtrates of *Stachybotrys atra*. This enzyme was readily separated during the course of purification from cellobiase as well as other enzyme activities which have often been attributed to β -glucosidases. This preparation appeared to be ideally suited for determining the specificities of a purified enzyme.

It was shown (Jermyn 1955*b*) that the *S. atra* β -glucosidase was inhibited by the glucose produced in the hydrolysis of aryl- β -glucosides but not by the aglycone. The absence of the aglycone inhibition that must be allowed for with some β -glucosidases (Veibel and Lillelund 1940), makes the task of determining the constants of competitive inhibitors of the enzyme much easier.

It has been emphasized by Levvy and Marsh (1954) that the problem of determining whether a given glycoside is a substrate of a certain glycosidase can be solved by observing whether it is a competitive inhibitor of the hydrolysis of a chromogenic substrate. This method has been successfully applied by Lederberg (1950) and Levvy and Marsh (1952). Ezaki (1940) considered the cross-inhibition of β -galactosidase by β -glucosides and β -glucosidase by β -galactosides in almond emulsin to prove them to be identical enzymes.

Ability to act as a competitive inhibitor is not confined to potential substrates, however; β -galactosidases are inhibited by galactonate or gluconate (Nishizawa 1942), taka-diaxase β -glucosidase by gluconates or glucono-1,4-lactone (Ezaki 1940), emulsin by the 1,4-lactone only (Horikoshi 1942) and rumen β -glucosidase by glucono-1,4- and -1,5-lactones (Conchie 1954).

The *S. atra* enzyme shares the property of being inhibited by glucose with many other β -glucosidases (Veibel and Eriksen 1940). The further compli-

cation of inhibition by excess substrate is also not uncommon in glycosidases and Nishizawa (1951) has figured a large number of examples for β -galactosidases. Hence the common simplified versions of the Michaelis-Menten theory of enzyme-substrate-inhibitor relationships must be applied to the glycosidases with considerable reserve.

II. MATERIALS

The glycosides required for this investigation were synthesized by suitable methods. Where they had been synthesized previously, their physical constants agreed with those in the literature. Jermyn (1955c) has described the synthesis and properties of those glycosides that were previously unreported.

The sugar acids required for inhibition experiments were synthesized as the potassium or calcium salts by the method of Moore and Link (1940).

The *N*-aryl-D-glucosylamines (*N*-glucosides) were recrystallized from water to give the monohydrate (*o*-toluidine, *p*-toluidine, 2 naphthylamine derivatives), or dihydrate (*p*-nitroaniline derivative) of the β -form.

The *Pseudomonas tumefaciens* polysaccharide was prepared using the method of Hodgson, Rijker, and Peterson (1945) and a culture of the bacterium given by Professor A. J. Rijker. The laminarin was a gift from Professor T. Dillon, and D-altrose and the heptoses were a gift from the late Professor C. S. Hudson.

The enzyme samples used, unless otherwise stated, were lead-precipitated fractions (Jermyn 1955a) with activity of at least 2000 units/mg N. It was found that the shape of the pH-activity curve (Jermyn 1955b), the Michaelis constants for the hydrolysis of salicin and *p*-nitrophenyl- β -glucoside, and the ratio of the hydrolysis rates of the two substrates were constant from one preparation to another.

III. METHODS

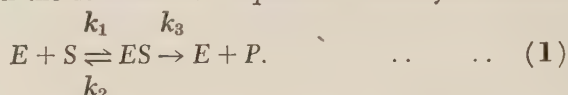
(a) *Michaelis Constants*

It was shown for four aryl- β -glucosides (phenyl-, *p*-nitrophenyl-, *o*-cresyl-, 2-naphthyl-) that K_m at pH 5 (citric acid-sodium phosphate buffer) and 28°C was independent of enzyme concentration. The concentrations of substrate and enzyme were therefore chosen to give a suitable optical density by the methods of determining phenol liberation previously described (Jermyn 1955a) as well as a marked dependence of enzyme activity on substrate concentration. For alkyl glucosides the liberated glucose was determined colorimetrically by the Somogyi-Nelson method (Nelson 1944). The lower limit of practical substrate concentration was taken as $2 \times 10^{-4}M$; below this point substrate concentration would change sufficiently during incubation to affect the results. Since excess substrate did not significantly inhibit at concentrations less than about $K_m \times 10^2$, this left a usable range of substrate concentrations for determining even the lowest K_m values.

The Michaelis constant was determined graphically according to the method of Lineweaver and Burk (1934), the slope of the lines being determined by the method of least squares.

(b) Determination of V_{max}

From the Lineweaver-Burk plot it is possible to calculate V_{max} , the theoretical velocity at infinite substrate concentration. This is in fact fictitious where excess substrate inhibits, but its value at a standard enzyme concentration can be used as a measure of k_3 in the fundamental equation for enzyme action



No attempt has been made to determine absolute values for k_3 and the various values of V_{max} were recalculated to moles of aglycone liberated during identical reaction periods at identical enzyme concentrations and expressed on a comparative basis with V_{max} for phenyl- β -glucoside as unity. The values are thus in fact though not in form comparable with the k_3 values of Veibel and Lillelund (1940).

(c) Determination of Inhibitor Constants

For a suitable concentration of inhibitor and enzyme the velocity of the inhibited and uninhibited hydrolysis of *p*-nitrophenyl- β -glucoside was determined over the substrate range 2×10^{-3} – 2×10^{-4} M and the results expressed as Lineweaver-Burk plots.

Where inspection of the graphs showed that the inhibition was of simple competitive type, K_i (inhibitor constant) was determined according to the principles set out by Massart (1950).

(d) Determination of Absolute Specificity

If 5 or 10 ml of a solution of an aryl-glycoside is incubated for 24 hr with 10 units of enzyme at 28°C, a rate of decomposition 10^{-4} times that of *p*-nitrophenyl- β -glucoside should give a marked liberation of the corresponding phenol, and a rate of 10^{-5} can be readily detected by sufficient attention to detail. No glycoside has been classified as a non-substrate if hydrolysis could be detected by this test. Since low reaction velocity is usually due to low affinity (as shown by a high K_m) rather than a low value of k_3 , the substrate concentration was usually kept at the highest level allowed by its solubility and this concentration is given with the listed non-substrates.

IV. ABSOLUTE SPECIFICITY OF THE ENZYME

Table 1 lists a number of glycosides which have been tested and found not to be substrates for the enzyme. Only compounds of types which have been claimed from time to time to be hydrolysed by β -glucosidases (cf. the review of Veibel 1950) were investigated. D-Glucofuranosides and L-glucosides have not been investigated in view of the results of Tsou and Seligman (1952)

with β -glucuronidase and 2-naphthyl- β -D-glucopyruronoside and β -D-glucufururonoside and the stereo-specificity of sugars and sugar acids as inhibitors of the *S. atra* β -glucosidase.

TABLE 1
NON-SUBSTRATES OF THE β -GLUCOSIDASE OF *S. ATRA*

Potential Substrate	Maximum Concentration Tested	Linkage (all pyranoside unless stated)
<i>p</i> -Nitrophenyl- α -D-glucoside	5×10^{-2} M	α -Glucoside
<i>p</i> -Nitrophenyl- α -D-mannoside	5×10^{-3} M	α -Mannoside
<i>p</i> -Nitrophenyl- β -D-mannoside	3×10^{-2} M	β -Mannoside
Phenyl- α -D-galactoside	3×10^{-2} M	α -Galactoside
<i>p</i> -Nitrophenyl- β -D-galactoside	5×10^{-2} M	β -Galactoside
Menthyl- β -D-glucuronide	5×10^{-2} M	β -Glucuronide
Maltose	2×10^{-3} M	α -Glucoside
Trehalose	10^{-1} M	α -Glucoside
Turanose	2×10^{-3} M	α -Glucoside
Sucrose	10^{-1} M	α -Glucoside
Cellulose	2×10^{-3} M	β -Fructofuranoside
Gentiobiose	2×10^{-3} M	β -Glucoside
Lactose	2×10^{-3} M	β -Galactoside
Melibiose	2×10^{-3} M	α -Galactoside
<i>p</i> -Nitrophenyl- β -cellobioside	5×10^{-2} M	$2 \times \beta$ -Glucoside
<i>p</i> -Nitrophenyl- β -maltoside	5×10^{-2} M	α -Glucoside
<i>p</i> -Nitrophenyl- β -lactoside	5×10^{-2} M	β -Glucoside
Phenyl- α -cellobioside	3×10^{-2} M	β -Glucoside
Phenyl- α -lactoside	5×10^{-2} M	β -Galactoside
Phenyl- α -maltoside	5×10^{-2} M	α -Glucoside
Cellodextrins	1%	β -Glucoside
Laminarin	1%	α -Glucoside
<i>Pseudomonas tumefaciens</i> polysaccharide	1%	β -Galactoside
		$2 \times \alpha$ -Glucoside
		Polymeric β -glucoside (1, 4)
		Polymeric β -glucoside (1, 3)
		Polymeric β -glucoside (1, 2)

V. INHIBITION OF THE ENZYME

(a) Screening for Effective Inhibitors

A number of lines of evidence showed that the enzyme is sensitive to the presence of aliphatic polyhydroxy compounds. For most of the substances tested the effect takes the form of a slight activation rising to a peak at intermediate concentrations and giving way to inhibition at lower concentrations (Fig. 1).

After studying a number of cases it was decided to introduce an empirical criterion in testing a large number of possible inhibitors for their effectiveness.

TABLE 2

EFFECT OF VARIOUS SUBSTANCES ON THE HYDROLYSIS OF 10^{-4} M *p*-NITROPHENYL- β -GLUCOSIDE
BY THE *S. ATRA* β -GLUCOSIDASE (pH 5, 28°C)

The limit of the experimental variation is about ± 3 per cent. All additions of ionic compounds have been accompanied by readjustments to the correct pH

Reagent	Percentage of Original Activity at Indicated Concentration of Reagent	
	10^{-2} M	10^{-1} M
Inhibitors		
D-Glucose	6	
Methyl- α -D-glucoside	93	58
Methyl- β -D-glucoside	87	29
2-Chloroethyl- β -D-glucoside	48	
<i>t</i> -Butyl- β -D-glucoside	25	
<i>cyclo</i> Hexyl- β -D-glucoside	12	
Phenyl- β -D-glucoside	96	
<i>p</i> -Nitrophenyl- α -D-glucoside	87	80
<i>o</i> -Hydroxymethylphenyl- β -D-glucoside (salicin)	40	
<i>p</i> -Hydroxyphenyl- β -D-glucoside (arbutin)	22	
D-Gluconate	16	
D-Glucono-1,5-lactone	0	
D-Mannose	23	
<i>p</i> -Nitrophenyl- α -D-mannoside	95	
D-Mannonate	69	
D-Galactose	89	35
2-Naphthyl- β -D-galactoside	95	
D-Galactonate	93	
D-Idose	91	
D- <i>gala</i> -L- <i>manno</i> Heptose	90	
D- <i>gala</i> -L- <i>gluco</i> Heptose	80	
D- <i>gulo</i> -L- <i>gala</i> Heptose	93	
D-Fructose	95	67
L-Sorbose	55	33
L-Rhamnose	92	
L-Rhamnonate	82	
L-Arabinose	65	19
D-Arabinose	90	40
D-Arabonate	32	
D-Arabono-1,4-lactone	40	
<i>p</i> -Nitrophenyl- β -D-xyloside	45	
D-Ribonate	92	
Maltose	87	
Phenyl- α -maltoside	66	
Phenyl- β -maltoside	68	
Maltobionate	69	
Cellobiose	85	
Phenyl- α -cellobioside	67	42
Cellobionate	58	
Lactobiono- δ -lactone	58	
Turanose	42	
Melezitose	90	55

TABLE 2 (Continued)

Reagent	Percentage of Original Activity at Indicated Concentration of Reagent	
	10 ⁻² M	10 ⁻¹ M
L-Menthyl- β -D-glucuronide	70	
Ascorbic acid	78	
<i>i</i> -Erythritol	93	
Ineffective		
L-Xylose	103	
D-Ribose	98	
Trehalose	102	
Dulcitol	98	111
Sorbitol	98	
Mannitol	99	
Methyl- α -D-mannoside	97	99
D-Glucuronate	99	
D-Glucurone	98	
D-Galactonate	101	
D-Galactono-1,4-lactone	101	
L-Arabinonate	101	
D-Xylónate	101	
D-Lyxonate	103	
D-Ribono-1,4-lactone	102	
D-Saccharate	99	
Mucate	99	
Lactobionate	97	
α -Glucoheptonate	98	
α -Glucoheptono-1,5-lactone	100	
Activators		
D-Xylose	108	
D-Lyxose	104	
Phenyl- β -lactoside	111	
Phenyl- α -lactoside	137	
<i>p</i> -Nitrophenyl- β -cellobioside	117	
Raffinose	108	
<i>i</i> -Inositol	114	
Ribitol (adonitol)	117	
Glycerol	125	
D-Altrose	114	
D-Glucosamine	120	
<i>N</i> -Acetyl-D-glucosamine	113	
D- <i>gulo</i> -L- <i>talo</i> Heptose	118	
D- <i>gluco</i> -D- <i>gulo</i> Heptose	120	
D- <i>manno</i> -D- <i>gala</i> Heptose	117	
Phenyl- β -D-mannoside	118	
Phenyl- α -D-mannoside	123	
Phenyl- α -D-galactoside	117	
Melibiose	112	
Lactose	112	
Sucrose	107	90
<i>p</i> -Nitrophenyl- β -lactoside	121	

Conditions were so chosen that about 30-40 per cent. of a 10^{-4} M solution of *p*-nitrophenyl- β -glucoside was hydrolysed, and a parallel experiment was run

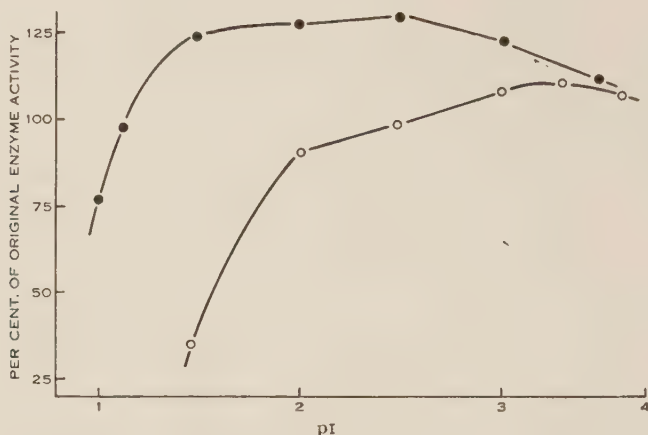


Fig. 1.—Effect of high concentrations of ○ sucrose and ● glycerol on the hydrolysis of 10^{-3} M *p*-nitrophenyl- β -glucoside at 28°C and pH 5.0 by *S. atra* β -glucosidase.

in which the solution was also 10^{-2} M in the compound under investigation. The results are set out in Table 2.

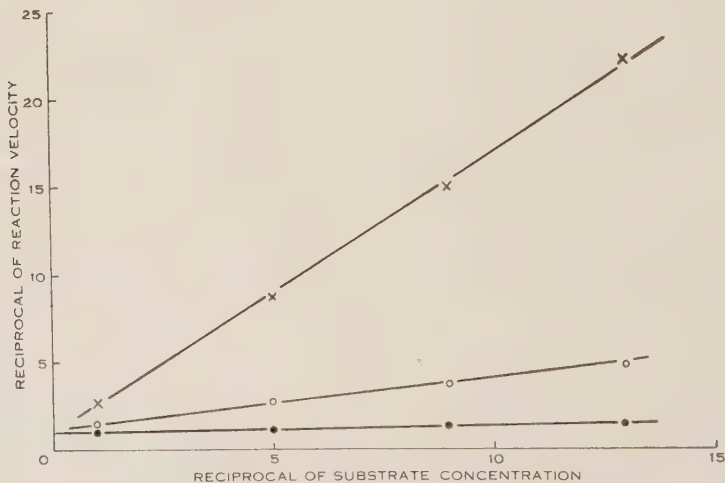


Fig. 2.—Arbutin (*p*-hydroxyphenyl- β -glucoside) and D-mannose as competitive inhibitors of the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase. ● No addition. ○ 10^{-2} M Mannose. × 10^{-2} M Arbutin. Unit substrate concentration = 2×10^{-3} M.

Since it was not possible to examine in detail all substances with an effect on the enzymic reaction, a certain number of selected cases have been used in an attempt to elucidate the mechanism of inhibition.

(b) *Competitive (Type 1) Inhibitors*

The sugars D-glucose, D-mannose, and D-arabinose, the sugar acids D-gluconic and D-arabonic, and the lactones D-glucono-1,5-lactone, D-arabono-1,4-lactone, and lactobionono- δ -lactone are sufficiently effective competitive inhibitors of the hydrolysis of *p*-nitrophenyl- β -D-glucoside for the competitive effect to be studied in isolation from non-competitive effects. Non-chromogenic β -glucosides as well as certain other glycosides which are not substrates but have the same ring configuration as β -D-glucosides (*p*-nitrophenyl- β -D-xyloside and menthyl- β -D-glucuronide) are also competitive inhibitors.

Figure 2 shows that both D-mannose and the β -glucoside arbutin give the typical Lineweaver-Burk plots to be expected for competitive inhibition.

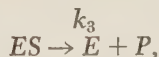
(c) *Type 2 Inhibition*

The plots of enzyme activity against inhibitor concentration for D-glucose (competitive inhibitor) and turanose are almost indistinguishable (Fig. 3). Yet when a Lineweaver-Burk plot is constructed for turanose inhibition (Fig. 4) it is apparent that it does not follow the criteria usually laid down for either competitive or non-competitive inhibition (cf. Massart 1950). For competitive inhibition $1/V_{\max}$ remains constant and the slope of the line and hence the apparent K_m are increased by the factor $(1 + [I]/K_i)$, where $[I]$ is the concentration of inhibitor; for non-competitive inhibition $1/V_{\max}$ and the slope of the line are increased by $(1 + [I]/K_i)$ and the apparent K_m remains constant. In type 2 inhibition, to the first approximation, $1/V_{\max}$ and the apparent K_m are both increased by the same factor and the slope of the line remains constant.

In terms of the constants of equation (1) it can be shown that

$$K_m = \frac{k_2 + k_3}{k_1} \quad \dots \quad (2)$$

The usual treatment of inhibition is based on the assumption that $k_2 \gg k_3$ and that k_3 can in practice be neglected. But if, on the other hand, $k_3 \gg k_2$ and the effect of a non-competitive inhibitor bound to the enzyme molecule is to block the stage



reducing the true k_3 to a new apparent value k'_3 for a given concentration of inhibitor, we have

$$K_m = \frac{k_2 + k_3}{k_1} \simeq \frac{k_3}{k_1},$$

and

$$K'_m = \frac{k_2 + k'_3}{k_1} \simeq \frac{k'_3}{k_1}.$$

This would lead to V_{\max} and apparent K_m decreasing in approximately the same ratio. Any discrepancy can be used to calculate the ratio of k_3 to k_2 ; for the hydrolysis of *p*-nitrophenyl- β -D-glucoside the ratio k_3/k_2 is at least 50. The likeness of the two curves in Figure 3 may therefore be interpreted as being due to the fact that they are both dissociation curves, one for the binding of

glucose at the enzymatically active centre, and one for the binding of turanose at a second centre. The affinity constant for turanose is about $5 \times 10^{-3}M$. Since the dissociation curve tends asymptotically to 100 per cent. inhibition at high

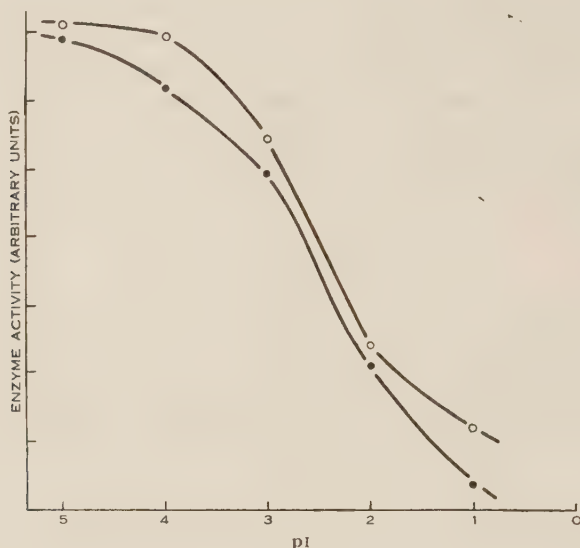


Fig. 3.—Effect of ○ turanose and ● glucose on the enzymic hydrolysis of $10^{-3}M$ *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase.

turanose concentrations, it appears that the reaction $ES \rightarrow P$ is totally blocked by turanose binding and not depressed to a lower rate.

TABLE 3
DETERMINATION OF K_i FOR GLUCOSE FROM THE DATA OF FIGURE 3

[I]	Slope	$\frac{\text{Slope of Inhibited Reaction}}{\text{Slope of Non-Inhibited Reaction}}$	$\frac{K_i}{[I]}$	K_i
Nil	0.096			
$10^{-4}M$	0.149	1.55	0.55	$1.8 \times 10^{-4}M$
$10^{-3}M$	0.61	6.3	5.3	$1.9 \times 10^{-4}M$
$10^{-2}M$	5.8	60	59	$1.7 \times 10^{-4}M$
$10^{-1.5}M$	13.7	142	141	$2.2 \times 10^{-4}M$
$10^{-1}M$	53.7	558	557	$1.8 \times 10^{-4}M$

Phenyl- α -cellobioside, methyl- α -D-glucoside, and L-arabinose give results similar to those for turanose.

It appears that inhibition by excess substrate is due to binding at the

second centre. This hypothesis can be tested by observing the consequences of counteracting high concentrations of a competitive inhibitor by raising the substrate concentration. The results for glucose and *p*-nitrophenyl- β -glucoside are shown in Figure 5. If the fact that all the lines do not converge to a common value of $1/V_{\max}$ is neglected and K_i is calculated from the ratio of slopes, the results tabulated in Table 3 are obtained. The constancy of K_i shows that the competition of glucose and *p*-nitrophenyl- β -glucoside for the enzymatically active centre is not affected by the action of excess substrate in depressing the activity.

Lineweaver-Burk plots corresponding to the atypical non-competitive inhibition here called type 2 have often been observed (e.g. for the inhibition of adenosinetriphosphatase by ethylurethane (Gross 1954)) without comment on the fact that they imply unusual relationships among the rate constants.

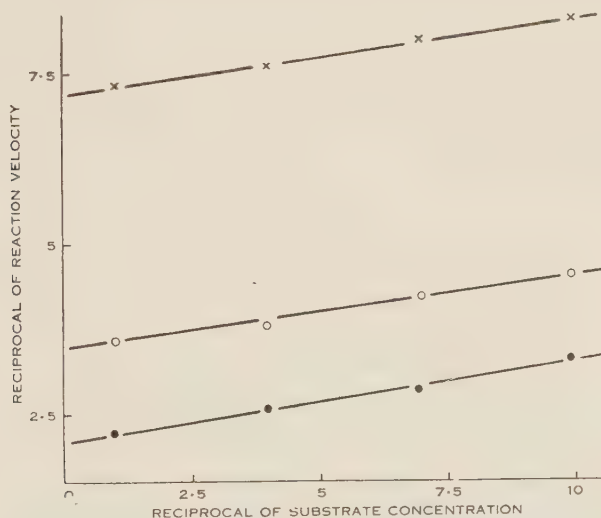


Fig. 4.—Effect of turanose on the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase. ● No addition. ○ 2×10^{-3} M Turanose. × 10^{-2} M Turanose. Unit substrate concentration 10^{-3} M.

(d) Mixed Type 1 and Type 2 Inhibition

If a substance has affinity for both binding centres on the enzyme molecule it can be expected to show behaviour intermediate between those of type 1 and type 2 inhibitors. D-Galactose gives Lineweaver-Burk plots that can be explained on this hypothesis (Fig. 6). The calculated K_i for galactose is found to have a constant value of 10^{-2} M with changing galactose concentration. The affinity constant for the second centre is about 5×10^{-1} M. This behaviour should be shown most clearly where, as for galactose, both constants are relatively high. Cellobiose behaves similarly to galactose with an even higher

value of K_i ; a number of effective β -glucosidic competitive inhibitors show a slight affinity for the second centre.

(e) Type 3 Inhibition

Substances giving curves such as those shown in Figure 1 are classified as type 3 inhibitors. It can readily be shown from a study of Lineweaver-Burk plots constructed for various concentrations of the reagent that the activating effect is the primary one, and that at higher reagent concentrations a competitive inhibition is superimposed on the activation. Glycerol was most intensively studied as a type 3 inhibitor but very similar results are given by sucrose. Figure 7 shows the effect of increasing concentration of glycerol, and it can be calculated that K_i for glycerol is approximately $2 \times 10^{-1}M$. For substances having rather smaller values of K_i , the results of Figure 8 are obtained.

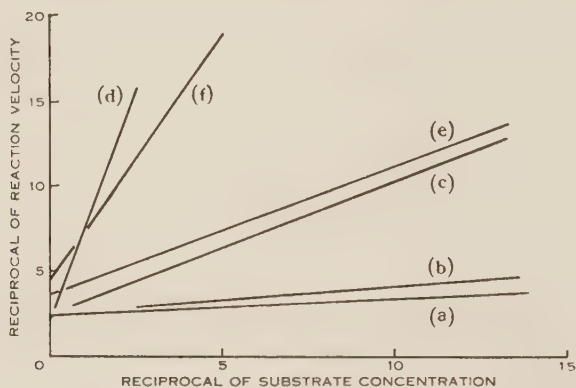


Fig. 5.—Effect of various concentrations of D-glucose on the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase. (a) No addition. (b) $10^{-4}M$ Glucose. (c) $10^{-3}M$ Glucose. (d) $10^{-2}M$ Glucose. (e) $10^{-1.5}M$ Glucose. (f) $10^{-1}M$ Glucose. Enzyme concentration constant. Unit substrate concentration = $10^{-3}M$ for (a)–(d); $2 \times 10^{-2}M$ for (e), (f).

Increasing the concentration of glycerol eventually leads to Lineweaver-Burk plots analogous to those of Figure 8, but there is a region of anomalous results between 0.5M and 1.65M. Here the degree of activation apparently falls as the glycerol concentration rises, leading finally for 1M and 1.5M glycerol to what may be described as “competitive activation” (Fig. 9). There is then a sharp change between 1.5M and 1.65M to the expected type of line, and calculation from the lines for 1.75M and 2M shows that the K_i ($2 \times 10^{-1}M$) is the same as for $10^{-1}M$ glycerol. In this range the dependence on concentration is so critical that it was impossible to reproduce the results exactly in successive experiments with glycerol solutions that had been made up separately. No hypothesis explaining these phenomena can be offered at present; it may be that the identity of K_i at high and low glycerol concentrations is illusory.

(f) Identity of Type 2 and Type 3 Effects

The Lineweaver-Burk lines for $2 \times 10^{-3}\text{M}$ turanose (Fig. 4) and 10^{-2}M glycerol (Fig. 7) are identical except that one is above and one below the null line. There is nothing to contradict the assumption that both effects are due to binding at the same centre, one reagent blocking the process $ES \rightarrow P$ and the other facilitating it. If this is true, V_{\max} should increase with increasing concentration of a type 3 reagent and this is found to be the case. Since the theoretical value of k_3 for complete saturation of the enzyme is unknown it is not possible to use such data to construct dissociation curves.

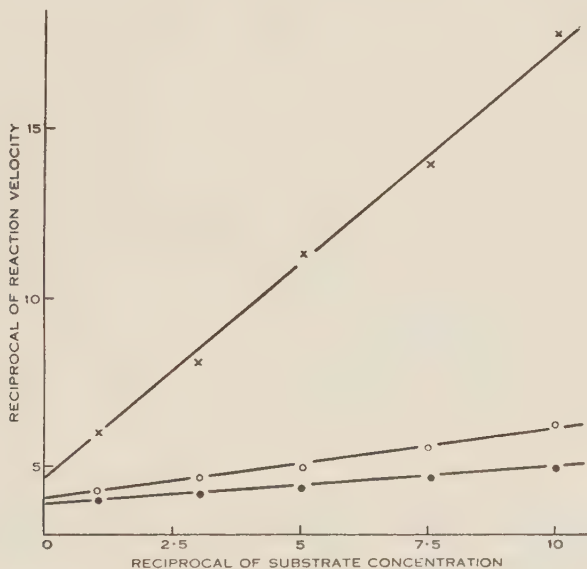


Fig. 6.—Effect of D-galactose on the hydrolysis of *p*-nitro-phenyl- β -glucoside by *S. atra* β -glucosidase. ● No addition. ○ 10^{-2} Galactose. × 10^{-1}M Galactose. Unit substrate concentration 10^{-3}M .

Most substances tested that are not competitive inhibitors show type 3 effects. Type 2 effects are only shown by substances with α -glucosidic linkages (turanose, phenyl- α -cellobioside, and methyl- α -glucoside), the sterically related D-galactose and L-arabinose (differing only by a $-\text{CH}_2\text{OH}$ attached to C_5 of the pyranose ring), and some β -glucosides.

VI. RELATION OF β -GLUCOSIDASE, β -XYLOSIDASE, AND β -CELLOBIOSIDASE

The non-identity of these three activities was demonstrated by the existence of batches of enzyme with only β -glucosidase activity, and by the variation in the ratio between the activities in batches containing all three (Table 4). Nevertheless, the activities must have belonged to very similar enzyme proteins

TABLE 4

COMPARISON OF THE ACTIVITY OF THREE PREPARATIONS OF *S. ATRA* β -GLUCOSIDASE IN HYDROLYSING 10^{-3} M *p*-NITROPHENYL- β -GLYCOSIDES AT 28°C AND pH 5 (CITRATE-PHOSPHATE BUFFER)

Batch No.	Rate of Hydrolysis of <i>p</i> -Nitrophenyl- β -cellobioside (β -xylosidase = 1)	Rate of Hydrolysis of <i>p</i> -Nitrophenyl- β -glucoside (β -xylosidase = 1)
2 (lead fractionated)	7.9	133
4 (lead fractionated)	1.6	107
5 (dialysed ethanol concentrate)	22.8	104
5 (after lead fractionation)	28.3	126
9*	1.7	157

* Fraction 3 (Jermyn 1955a).

since the lead-fractionation procedure which sufficed to separate all other enzymes from the β -glucosidase did not much affect the activity ratios.

Inspection of the pH-activity curves for the three shows them to be rather unlike (Fig. 10) although similar variation in the pH-activity curves for a single enzyme against different substrates has often passed without comment.

A number of possible inhibitors were then tried with the results shown in Table 5. From the results recorded in the last section, it appears that they

TABLE 5

COMPARISON OF CERTAIN SUBSTANCES AS INHIBITORS OF THE β -GLUCOSIDASE, β -CELLOBIOSIDASE, AND β -XYLOSIDASE IN A SINGLE BATCH OF *S. ATRA* ENZYME (LEAD-FRACTIONATED BATCH 5 OF TABLE 4). INHIBITORS, 10^{-2} M; *p*-NITROPHENYL- β -GLYCOSIDE SUBSTRATE, 10^{-4} M

Inhibitor	Per Cent. of Uninhibited β -Glucosidase	Per Cent. of Uninhibited β -Cellobiosidase	Per Cent. of Uninhibited β -Xylosidase
D-Glucose	10	21	4
Phenyl- α -D-glucoside	96	120	
<i>p</i> -Nitrophenyl- α -D-glucoside	89	113	
Arbutin	13	10	33
D-Mannose	34	25	
Phenyl- α -D-galactoside	117	116	
2-Naphthyl- β -D-galactoside	95	120	
D-Xylose	109	122	81
<i>p</i> -Nitrophenyl- β -D-xyloside	45	85	
Cellobiose	86	9	
Cellobionate	58	7	
Phenyl- α -cellobioside	72	29	
<i>p</i> -Nitrophenyl- β -lactoside	121	144	
Lactobionate	97	94	
Maltobionate	69	29	

would be unlikely to be conclusive without detailed investigation of inhibitor type and the relative affinities of substrates and competitive inhibitors. Taken together the three lines of evidence (Table 4, Fig. 10, Table 5) would suggest that there were three separate enzymes in the purified preparation from batch 5, but fall far short of proof.

In batch 6 there was no detectable β -xylosidase or β -cellobiosidase, thus proving the β -glucosidase to be a separate entity.

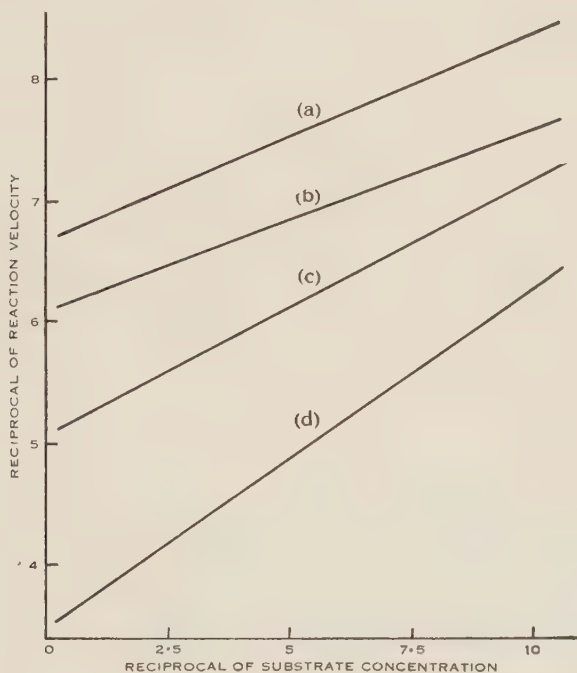


Fig. 7.—Effect of various concentrations of glycerol on the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase. (a) No addition. (b) 10⁻²M Glycerol. (c) 10^{-1.5}M Glycerol. (d) 10⁻¹M Glycerol. Unit substrate concentration 10⁻³M.

VII. RELATIVE SPECIFICITY OF THE ENZYME

A large number of β -glucosides was examined as substrates for the enzyme and competitive inhibitors of the hydrolysis of *p*-nitrophenyl- β -D-glucoside. The values of K_m , relative V_{max} , and K_i are shown in Table 6, together with K_i values for a number of those non-substrates that were shown to be competitive inhibitors.

The thiophenol liberated from phenyl- β -thiogluconide could be readily estimated by the Folin-Ciocalteu method and this glucoside shown to be a substrate as well as an inhibitor. Although the aryl-*N*-glucosides were effective

competitive inhibitors it was not possible to show whether or not they were substrates because of the high rate of spontaneous hydrolysis. With *N-p*-nitrophenyl- β -D-glucosylamine, which is much less absorptive than *p*-nitroaniline in the range 410-450 $m\mu$, the increase in optical density at 435 $m\mu$ (Beckmann spectrophotometer) was used as a measure of the hydrolysis of the *N*-glucoside. It was found that the rate of enzymic hydrolysis of this substrate at $10^{-3}M$ could not exceed 10^{-4} times that of *p*-nitrophenyl- β -D-glucoside.

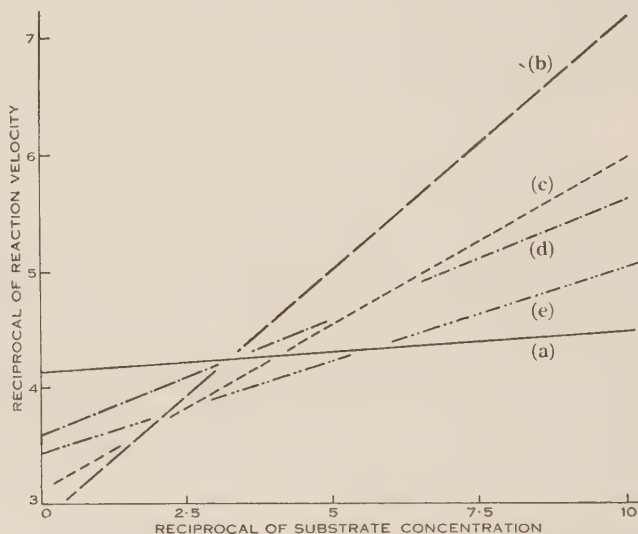


Fig. 8.—Combination of type 1 and type 3 effects in the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase. (a) No addition. (b) $10^{-2}M$ L-Sorbose. (c) $10^{-1}M$ D-Fructose. (d) $10^{-1}M$ Ascorbic acid. (e) $10^{-1}M$ Sucrose. Unit substrate concentration $2 \times 10^{-3}M$.

It is a consequence of the hypothesis developed to explain the behaviour of type 2 and 3 inhibitors that K_m and V_{max} might be expected to show parallel variation, since the former is strongly influenced and the latter wholly determined by the value of k_3 . Since the relationship is

$$K_m \simeq \frac{k_3}{k_1},$$

and the relative effects of different substrates on k_1 and k_3 are unknown, strict proportionality is not to be expected. Figure 11 shows that the trend is in fact in the opposite direction to that predicted and the substrate effect on k_1 must outweigh that on k_3 .

When L-arabinose, a type 2 inhibitor, was tested for its effect on the hydrolysis of a number of β -glucosides of high K_m , it was found that Lineweaver-Burk plots were obtained identical in form with those of Figure 4. It appears that $k_3 \gg k_2$ here also, and that the kinetics of all substrates investigated can be explained in terms of k_3 and k_1 only. It would be expected from the explana-

tion advanced for type 2 inhibition, that V_{\max} would be diminished in the same proportion for all substrates at a given concentration of L-arabinose, and this was found to be the case.

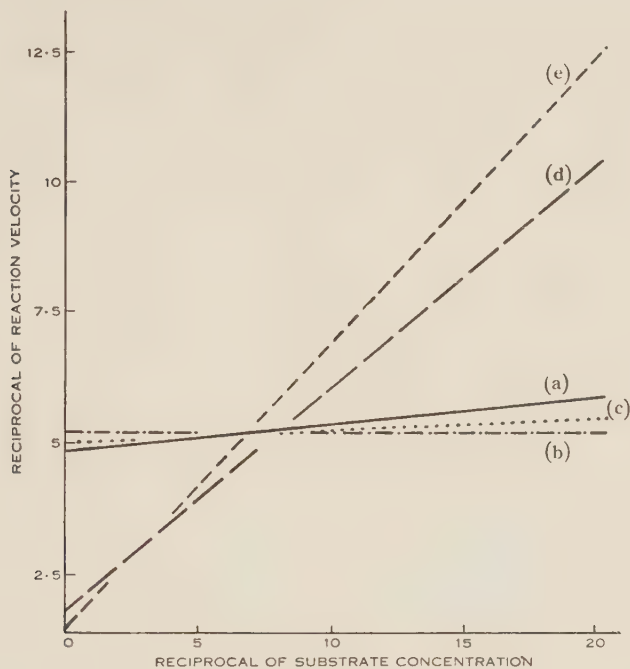
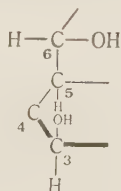


Fig. 9.—Effect of higher concentrations of glycerol on the hydrolysis of *p*-nitrophenyl- β -glucoside by *S. atra* β -glucosidase. (a) No addition. (b) 1.0M Glycerol. (c) 1.5M Glycerol. (d) 1.75M Glycerol. (e) 2.0M Glycerol. Unit substrate concentration 2×10^{-3} M.

VIII. DISCUSSION

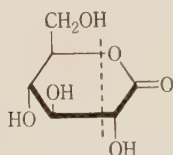
(a) Absolute Specificity of the Enzyme

One portion of the specificity of the *S. atra* β -glucosidase appears to be directed towards the configuration about carbon atoms 3, 5, and 6 of glucose and the required structure appears to be given by

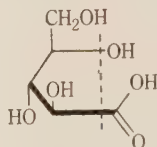


All those competitive inhibitors of the enzyme which do not contain β -glucosidic linkages appear to fit into this pattern. The most important single element of this structure appears to be that the configuration at C_3 should be

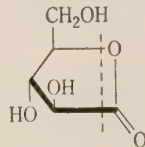
the same as that in D-glucopyranose and no sign of activity as a competitive inhibitor has been found in any substance with the opposite configuration. Gottschalk (1950) predicted from a survey of the relevant data that one point of specific attachment of β -glucosides to all β -glucosidases would prove to be the $-\text{OH}$ group at C_3 . The pyranose or any other ring structure does not seem to be necessary for activity; thus, although the activity of D-gluconic acid may be explained by the small amount of D-gluconolactone in equilibrium with it at pH 5 (cf. the results of Conchie 1954) this can hardly be the case with D-arabonic acid and D-arabonolactone. Some examples of inhibitors with the full D-glucose structure on the critical carbon atoms are given below.



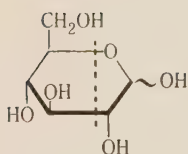
D-Glucono-
1,5-lactone
 $K_i = 0.32 \times 10^{-5}\text{M}$.



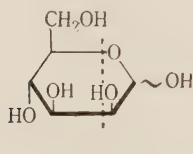
D-Arabonic acid
 $K_i = 4.6 \times 10^{-5}\text{M}$.



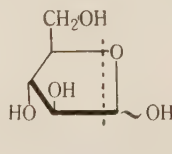
D-Arabetolactone
 $K_i = 27 \times 10^{-5}\text{M}$.



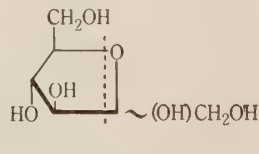
D-Glucopyranose
 $K_i = 19 \times 10^{-5}\text{M}$.



D-Mannopyranose
 $K_i = 43 \times 10^{-5}\text{M}$.



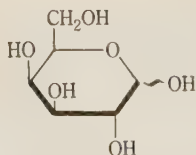
D-Arabinofuranose
 $K_i = 580 \times 10^{-5}\text{M}$.



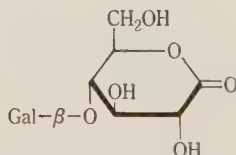
D-Fructofuranose
 $K_i = 1000 \times 10^{-5}\text{M}$.

In two cases (D-fructose, D-arabinose) the equilibrium in solution is against the forms with the structure required for activity, and the K_i values found experimentally are not of the same order as those of the true inhibiting species. Similarly there is no indication from the data whether D-arabonic acid or the D-arabonate ion is the inhibiting species and the true K_i will again be different from the calculated value.

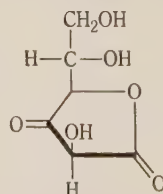
The configuration at C_4 does not appear to be critical and if the rest of the structure is correct variations at this centre still result in competitive inhibitors:



D-Galactopyranose
 $K_i = 1000 \times 10^{-5}\text{M}$.



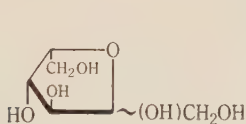
Lactobionolactone
 $K_i = 87 \times 10^{-5}\text{M}$.



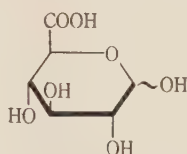
D-Ascorbic acid
(one keto form; not
favoured by equilibrium)
 $K_i = 140 \times 10^{-5}\text{M}$.

The insertion of a β -galactopyranosyl group at C_4 in the very active D -glucono-1,4-lactone molecule gives a fairly active inhibitor; in the less active D -glucose molecule it leads to a disaccharide (lactose) without detectable activity.

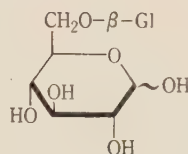
It appears that the nature rather than the configuration of the groups around C_5 is important, thus L -sorbitose is a competitive inhibitor, while D -glucuronic acid, D -glucurone, and gentiobiose ($6\text{-}\beta\text{-D-glucopyranosyl-D-glucose}$) are not.



L -Sorbofuranose
 $K_i = 87 \times 10^{-5}M$.

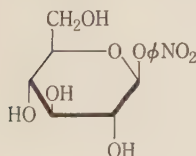


D -Glucuronic acid
(pyranose form)
non-competitive.

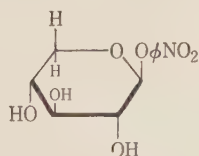


Gentiobiose
non-competitive.

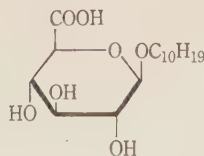
A second determinant of a specific attachment between the enzyme and the active molecule appears to be the configuration about C_1 (cf. the discussion in Gottschalk 1950). Neither D -glucuronic acid nor D -xylose are competitive inhibitors of the enzyme, but p -nitrophenyl- β - D -xylopyranoside is an inhibitor comparable with aryl- β - D -glucosides, and menthyl- β - D -glucopyruronoside is comparable with alkyl- β - D -glucosides. The specificity does not appear to require that the β -linkage shall be through oxygen; phenyl- β - D -thioglucoside is both a substrate and an inhibitor assuming that all the observed effects are due to a single enzyme. All other β -glucosidases which have so far been tested are unable to hydrolyse phenyl- β -thioglucoside (Veibel 1950), although Conchie (1954) showed it to be a slight inhibitor, not established as competitive, of rumen β -glucosidase. A linkage through nitrogen appears to allow attachment at the specific centre and the aryl- N - β -glucosides are competitive inhibitors, although it is doubtful whether they are substrates.



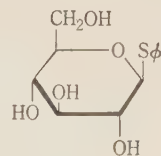
p -Nitrophenyl- β - D -glucopyranoside
 $K_m = 5 \times 10^{-5}M$.
Substrate.



p -Nitrophenyl- β - D -xylopyranoside
 $K_i = 3.7 \times 10^{-5}M$.
Non-substrate.



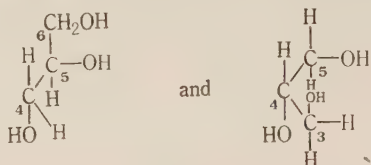
Menthyl- β - D -glucopyruronoside
 $K_i = 1200 \times 10^{-5}M$.
Non-substrate.



Phenyl- β - D -thioglucopyranoside
 $K_i = 153 \times 10^{-5}M$.
Substrate.

This requirement appears to involve the space relationship between the aglycone residue and the hydroxyl groups on C_2 and C_4 since neither mannosides nor galactosides are competitive inhibitors.

It would appear that while it is sufficient for a competitive inhibitor to meet one of the stereo-specificity requirements, a substrate must meet them both. There must also be a limit to the detection of any specific effects set by the apparent general affinity for polyhydroxyl compounds (K_i for glycerol, $20,000 \times 10^{-5}M$). Although glycerol can be written in such forms as



it is impossible to believe that its affinity for the enzyme can be any more specific than that of the many other polyhydroxyl compounds in which similar portions of the molecule occur. This effect does not appear to be limited to the *S. atra* β -glucosidase, or even to β -glucosidases in general, for de Grandchamp-Chaudun and Moreau (1953) found that besides the competitive inhibition of various sucrases by such sugars as glucose, fructose, and galactose with structures possibly related to the substrate, there was also a similar effect from glycerol at rather higher concentrations.

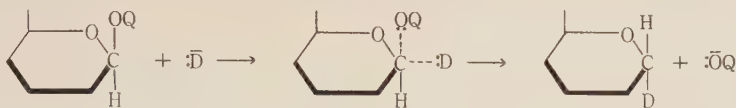
The specificity of the β -glucosidase of *S. atra* is much narrower than that of most β -glucosidases previously studied (Veibel 1950; Gottschalk 1950). If affinity for the enzymically active centre is considered without regard to whether the bound substance is a substrate or a competitive inhibitor, then the specificity of the enzyme resembles most closely that of brain hexokinase as reported by Sols and Crane (1954) whose specificity is directed towards the hydroxyl groups on C_1 , C_3 , C_4 , and C_6 of glucose. The most obvious differences are the lack of requirement for an intact pyranose ring, the actually enhanced affinity arising from replacement of CHOH by CO at C_1 (*D*-glucono-1,5-lactone is inactive for hexokinase) and C_4 , and the ineffectiveness of a nitrogen atom on C_2 (the glucosaminium ion and *N*-acetylglucosamine are not competitive inhibitors).

Crane and Sols (1954) also showed that there was a second binding centre on the brain hexokinase molecule leading to non-competitive inhibition of the enzymic reaction. Some elements of the specificity of the second binding centre of the β -glucosidase, at any rate for molecules blocking $ES \rightarrow E + P$, may be discerned in its affinity for α -glucosides and *D*-galactose (+ — — +) and *L*-arabinose (— — +). It may be speculated whether further research might not reveal it to be a second enzymically active centre with substances with affinity for the first centre acting as type 2 inhibitors.

(b) Relative Specificity of the Enzyme

Koshland (1954) and Koshland and Stein (1954) have developed and presented experimental evidence for a theory by which enzymes showing a high specificity for the *R* portion of the *R*-O-Q molecule and acting as *R*-transferases catalyse the fission of the *R*-O bond. The glucotransferase action of β -glucosidases is well known, and the *S. atra* β -glucosidase is highly specific for the glucose portion of the molecule, so that it is reasonable to follow Koshland and

Stein and write (the apparent absence of inversion about C_1 in the finally liberated hydrolysis product being actually a double inversion (Koshland 1953))



for the fundamental step in the action of β -glucosidase.

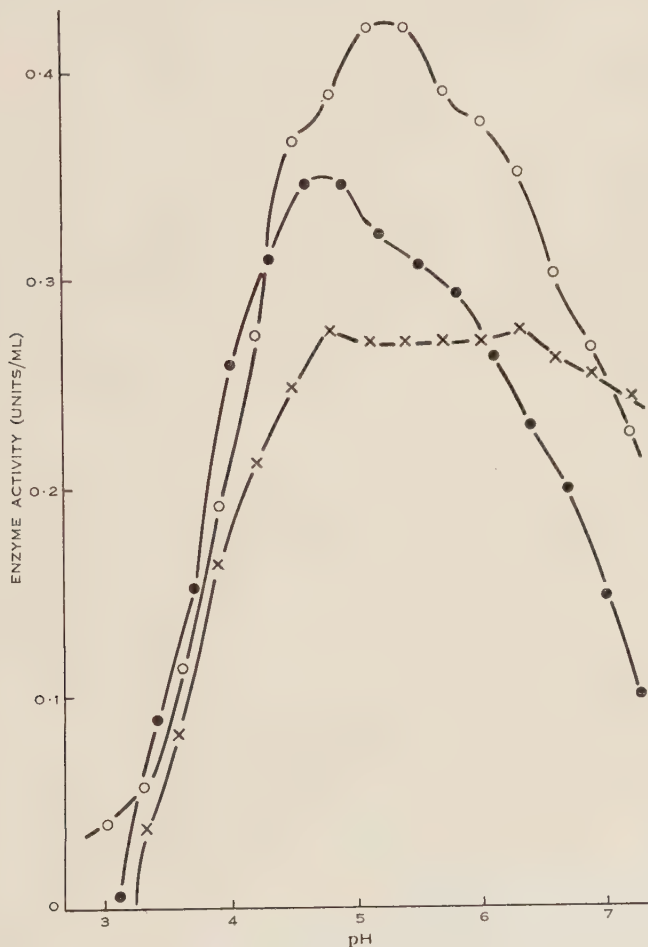


Fig. 10.—pH-Activity curves in citric acid-sodium phosphate (McIlvaine) buffers for two other enzyme activities in a preparation of *S. atra* β -glucosidase. All activities measured against 10^{-3}M *p*-nitrophenyl- β -glycoside at 28°C . O β -Cellobiosidase. X β -Xylosidase. ● β -Glucosidase from a preparation without β -xylosidase or β -cellobiosidase activity.

The primary nucleophilic reagent $:\bar{D}$ is unspecified. The nature of Q can affect the rate of the reaction either by altering the electronic structure of the R-O bond, or through steric influences on the approach of electrophilic reagents

TABLE 6
RELATIVE SPECIFICITY OF THE β -GLUCOSIDASE OF *S. ATRA* FOR VARIOUS SUBSTRATES AND INHIBITORS

Reagent	K_m ($M \times 10^{-5}$)	Relative V_{max} (phenyl- β - glucoside = 1)	K_i for the Hydrolysis of <i>p</i> -Nitrophenyl- β -D- glucoside ($M \times 10^{-5}$)
β -D-Glucosides			
Phenyl-	7.2	1.00	33
<i>p</i> -Hydroxyphenyl-			7.6
<i>p</i> -Nitrophenyl-	5.0	1.05	
<i>m</i> -Nitrophenyl-	36	0.88	
<i>o</i> -Nitrophenyl-	*		
<i>p</i> -Methylphenyl-	9.5	0.52	17.0
<i>m</i> -Methylphenyl-	6.7	1.24	27.2
<i>o</i> -Methylphenyl-	66	0.32	217
<i>o,o'</i> -Dimethylphenyl-	c. 680	0.002	*
<i>o</i> -Hydroxymethylphenyl-	122	0.37	216
2- <i>iso</i> Propyl-4-methylphenyl-	c. 700	0.022	*
<i>o</i> -Methoxyphenyl-	9.2	1.36	43.9
<i>m</i> -Methoxyphenyl-	8.2	0.83	4.3
<i>p</i> -Methoxyphenyl-	5.1	0.74	4.4
<i>o</i> -Chlorophenyl-	34	0.38	22.8
<i>p</i> -Chlorophenyl-	4.3	1.23	9.6
2,4-Dichlorophenyl-	7.0	1.03	8.8
<i>o</i> -Iodophenyl-	33	0.04	28
<i>p</i> -Iodophenyl-	3.1	0.13	3.0
2,4-Di-iodo-6-methylphenyl-	*		
<i>p</i> -Phenylphenyl-	12	116	2.3
1-Naphthyl-	178	0.13	5.8
2-Naphthyl-	5.2	0.88	4.5
1-Methyl-2-naphthyl-	21	0.12	23.5
1-Bromo-2-naphthyl-	5.0	0.25	5.7
6-Bromo-2-naphthyl-	2.2	0.72	3.1
8-Quinoliny-	*		
cyclohexyl-	63	0.096	164
sec.-Butyl-	84	0.12	
isoPropyl-	1300	0.055	
tert.-Butyl-†		0.015	
2-Chloroethyl-†		0.016	
Methyl-†		0.035	10,000
<i>N</i> - β -D-Glucosides			
<i>N</i> - <i>o</i> -Tolyl- β -D-glucosylamine			105
<i>N</i> - <i>p</i> -Tolyl- β -D-glucosylamine			34
<i>N</i> -2-Naphthyl- β -D-glucosylamine			27
Phenyl- β -D-thioglucoiside	200	0.35	153
Sugars			
D-Glucose			19
D-Mannose			43
D-Galactose			c. 1,000

* Activity too low for accurate measurement.

† Non-linear Lineweaver-Burk plots at the substrate concentrations necessary for measurable activity; reaction velocities at $10^{-2}M$ have been used for comparison.

TABLE 6 (Continued)

Reagent	K_m ($M \times 10^{-5}$)	Relative V_{max} (phenyl- β - glucoside = 1)	K_t for the Hydrolysis of <i>p</i> -Nitrophenyl- β -D- glucoside ($M \times 10^{-5}$)
D-Fructose			c. 1,000
L-Sorbose			58
D-Arabinose			580
Sucrose			600
Cellobiose			5,900
Sugar acids and lactones			
D-Gluconic acid			140
D-Glucono-1,5-lactone			0.32
D-Arabonic acid			4.6
D-Arabeto-1,4-lactone			27
4-(β -D-galactopyranosyl)-D- Glucono-1,5-lactone (lactobiono- δ -lactone)			87
Others			
<p><i>p</i>-Nitrophenyl-β-D-xyloside</p>			3.7
Menthyl- β -D-glucuronide			1,200
Glycerol			c. 20,000
Ascorbic acid			140

(protons free or bound to the enzyme) to the R-O bond, and on the fit of the substrate to the enzyme surface. It may be predicted qualitatively that Q groups capable of acting as electron sinks will increase the reaction rate and, in agreement with this, k_3 for phenyl- β -glucoside is about 10 times greater than for cyclohexyl- β -glucoside (and other secondary alkyl- β -glucosides).

This prediction cannot be applied in detail, however, and if k_3 values for *para*-substituted phenyl- β -glucosides are compared, we find the order



which does not coincide with any series of electronic effects. It is apparent that a large number of factors are involved in the stability of the transition complex. It seems unnecessary, however, to postulate with Pigman (1944) that there are any specific forces of attraction between the aglycone and the enzyme; indeed with hydrocarbon radicals this seems most unlikely (cf. Gottschalk 1950). Where the aglycone is another sugar radical which would be expected to have some affinity for the enzyme, the reaction rate is zero for the *S. atra* enzyme.

The *S. atra* β -glucosidase agrees with the fungal β -glucosidases from *Aspergillus oryzae* and *A. niger* studied by Miwa *et al.* (1937) in hydrolysing *ortho*-substituted phenyl- β -glucosides much more slowly than the parent β -glucoside. This effect extends to 1-naphthyl-, 8-quinoliny-, and substituted 2-naphthyl- β -glucosides. V_{max} is diminished and K_m is increased, and since the effects do not always run in parallel, it is apparent that k_1 , k_2 , and k_3 are probably all affected by the *ortho*-substituent. It has already been shown (Fig. 11) that

the preponderating effect must be on k_1 , the rate of attachment of substrate to enzyme to form the active complex. One way in which this effect could arise is by restricting free rotation about the C-O-C bonds to allow the required fit of the glucoside to the enzyme surface and experiments with models show that rotation in 2,6-dimethylphenyl- β -glucoside is almost completely hindered. The order for the K_m of mono-*ortho*-substituted phenyl- β -glucosides ($-\text{OCH}_3 < \text{H} < \text{Cl}$, $\text{CH}_2\text{OH} < \text{CH}_3 < \text{I} < \text{CH}(\text{CH}_3)_2 < \text{NO}_2$) contains deviations from the accepted list of radical sizes and specific effects must be invoked here also.

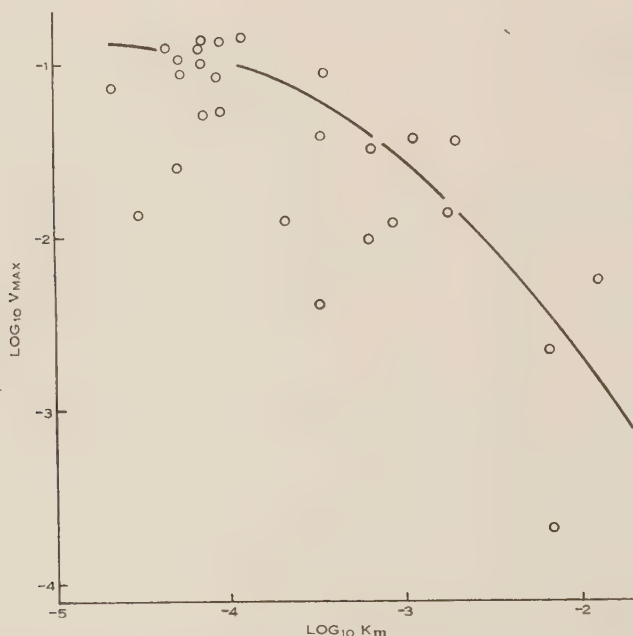


Fig. 11.—Relationship between the Michaelis constant (K_m) and relative V_{\max} (proportional to k_3) for substrates of *S. atra* β -glucosidase. The expected relationship from the equation $K_m \simeq k_3/k_1$ is that V_{\max} should increase as K_m increases, i.e. the trend line should lie from lower left to upper right. Each point gives the values (taken from Table 6) of K_m and relative V_{\max} for a single substrate.

The effect of 2,6-dimethyl and 2-iodo-6-methyl substitution can scarcely be due to other than steric causes, and this behaviour has been generally, but not so strongly, observed by other workers (Helferich and Scheiber 1934; Veibel and Shin-Lin Yang 1952). The *ortho* effect is also visible in the much smaller efficiency of *N*-*o*-tolyl- β -D-glucosylamine as a competitive inhibitor than the *p*-tolyl derivative.

In agreement with the conclusions set out by Veibel (1950) for a number of β -glucosidases the *S. atra* β -glucosidase hydrolyses *sec.*-alkyl- β -glucosides more rapidly than *prim.*- and *tert.*-alkyl- β -glucosides. This behaviour is shown not

only in a lower K_m but apparently in a higher V_{max} . The *prim.*- and *tert.*-alkyl- β -glucosides are in fact unsatisfactory substrates for study with the *S. atra* β -glucosidase since secondary effects interfere with any attempt to determine the Michaelis constant.

It will be seen that the specificities of the *S. atra* β -glucosidase represent no major departure from those recorded in the literature for related enzymes. For one departure from expected behaviour, however, no explanation can be offered at present. This is the non-equivalence of K_m for most of the phenolic β -glucosides with K_i for their competitive inhibition of the hydrolysis of *p*-nitrophenyl- β -glucoside (Table 6). These glucosides apparently fulfil the criteria for competitive inhibition (linear Lineweaver-Burk plots tending to a constant value of $1/V_{max}$, constant K_i with changes in $[I]$, and give linear Lineweaver-Burk plots when their K_m is determined directly. It is apparent that one or both of K_m and K_i are not true dissociation constants.

One conclusion that can be drawn from the observations made on inhibitors of the *S. atra* β -glucosidase is that decisions about enzyme specificity made after the study of competitive inhibition by the techniques reviewed by Levvy and Marsh (1954) need to be treated with some caution. Thus no substrate of the enzyme failed to act as a competitive inhibitor, but competitive inhibitors are not necessarily substrates (e.g. β -xylosides). Glucose is a competitive inhibitor and β -glucosides are substrates of the enzyme but, although mannose is an almost equally effective competitive inhibitor, neither α - nor β -mannosides are substrates.

IX. ACKNOWLEDGMENTS

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INHERITANCE OF ANTIBODY RESPONSE

I. SHEEP RED CELLS

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Summary

The heritability of antibody response to sheep red cells (S.R.C.) was found to be 0.2836 ± 0.1475 at 3 days and 0.1097 ± 0.1165 at 6 days. These estimates are subject to environmental variation of measurement which was found subsequently but could not be corrected for. An example of non-genetic, non-environmental error of measurement is illustrated experimentally and the consequences of this type of error are discussed.

I. INTRODUCTION

An approach to the study of the inheritance of the amount of antibody produced in response to injections of antigens has been made by Sang and Sobey (1954) and Sobey (1954). Where an apparent single antibody response to tobacco mosaic virus (T.M.V.) was measured in rabbits, the ability to produce antibodies in different amounts was found to be highly heritable ($h^2 = 0.87 \pm 0.09$), but the response to bovine plasma albumen (B.P.A.), a complex antigen demonstrated to be producing several antibody responses, showed no heritability ($h^2 = 0.09$). As the heritability of a character is an estimate of the fraction of all the variance of that character which is due to genetic differences, a smaller heritability could be due either to a lower genetic variance or to a greater non-genetic variance, or to both. Measurements of response to T.M.V. and to B.P.A. were made in each case with samples of the same serum from the same bleeding, thus one would not expect any differences in environmental variation. Why, then, should the heritability of response to B.P.A. be so much lower than that to T.M.V.?

The response to T.M.V. was measured by the "equivalence zone" technique which involves testing the supernatants for the presence of antigen and antibody after precipitation is complete. Where more than one antibody is concerned in the reaction, the equivalence zone is likely to be absent when the different responses are markedly dissimilar, or wide where they are less dissimilar. Both these phenomena were occasionally encountered and the response determined by interpolation (Sobey 1954), indicating that all animals do not react similarly to T.M.V. and in some cases appeared to produce more than one antibody response. Their occurrence was, however, too infrequent to mask the high heritability to T.M.V.

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The response to B.P.A. was measured by the "optimal proportions" method, and where measurements involving more than one antibody response are made using this technique, it seems quite probable that the index of response, i.e. the first tube to flocculate, will be of the antibody in highest concentration whose optimal coincides with that antigen-antibody ratio. With multiple antibody responses one might, *a priori*, expect more than one optimal point in a series of antigen dilutions. These were rarely found, but the arguments put forward by Elek (1949) suggest that they are not necessarily an invariable occurrence. Further, if flocculation is a second stage of the precipitin reaction, and less specific than the first stage of actual combination of antigen and antibody (Boyd 1947; Topley and Wilson 1948), initiation of flocculation by the antibody in highest concentration could quite conceivably mask any secondary optima. This inadequacy of the measure of response could result in an apparent lack of genetic variability. It is impossible to determine which antibody response among those present is being measured in any one animal, and this could result in a similar measure of response being determined for different antibodies in different animals.

The haemolytic titre, which is used in the work to be described, is probably open to limitations similar to those envisaged for the optimal proportions technique, where more than one antibody response is concerned. By serial dilution of the antiserum, the antibodies with lower responses will quickly become too low in concentration to contribute towards haemolysis, and the end-point giving the measure of response will be that of the antibody which is in highest concentration. On this view, the different antibody responses are very unlikely to be additive in respect of the end-point. The consequences of this, as with the optimal proportions method, would be that in no instance would total antibody response be measured. The measure would actually be that of the antibody in highest concentration, and since the same antibody would not necessarily be present in greatest amount in all individuals, it would not measure the variation of a single specific antibody response either. This could result in an apparent lack of genetic variability.

In order to check this point, experiments were designed to measure the heritability on a second complex antigen and to examine possible reasons for low heritability, if this were found.

II. EXPERIMENT 1

(a) Materials

In all, 120 pairs of albino mice were chosen at random from the second generation of a line of mice selected for sensitivity to oestrogen. These constituted the parents and varied between 19 and 30 weeks in age with an average of 26 weeks at the time of testing. Of these, 114 pairs produced litters, and four offspring, two of each sex where possible, were kept from each litter at the time of weaning. The offspring varied between 19 and 25 weeks of age with an average of 22 weeks at the time of testing. The antigen selected was sheep erythrocytes (S.R.C.) and all the S.R.C. used were obtained from the same sheep.

(b) Methods

Sheep blood was collected from the jugular vein into dextrose-citrate (3 per cent. disodium citrate, 3 per cent. dextrose in distilled water, 1.5 ml to 10 ml blood), stored at 4°C, and always used within 6 days of collection. Before use, S.R.C. were washed three times and suspended in Ca-Mg saline (Mayer *et al.* 1946).

S.R.C. was injected into a tail vein using a ½-in., 26 gauge needle and a tuberculin syringe. All injections were of 0.5 c.c. The method of bleeding was adapted from one devised by Dr. J. Bullen of Cambridge (personal communication). Prior to bleeding, the body temperature of the mice was raised until their tail veins became distended. They were then lightly anaesthetized and the tip of the tail (about 1 cm for mice not previously bled, and 2 or 3 mm for others) amputated with a pair of sharp scissors. The stump was quickly lowered into a labelled Wasserman tube contained in a larger glass tube under a negative pressure of 10 cm Hg. Tubes containing blood were allowed to stand at room temperature overnight, the serum pipetted into clean tubes, and centrifuged to remove any residual cells, pipetted into storage tubes, and stored at -20°C. This method allows mice to be bled without mortality at the rate of one every 2 min, with an average yield of 0.25 ml serum per bleeding.

Guinea pig serum was used throughout as a source of complement. Guinea pigs were bled by cardiac puncture, the blood allowed to clot at room temperature over 3 hr, and the serum removed, centrifuged, and stored in 2-ml amounts at -20°C. Complement was made up of the pooled serum from 20 male animals selected by previous testing for their capacity to yield high-titre complement. Three M.H.D. were used in all antibody titrations. Complement was never stored for periods exceeding 4 weeks and repeated titrations showed no decrease in titre during this period. Material thawed but not used on the day of test was discarded.

Serum was inactivated at 56°C for ½ hr on the day of test. Using a pipette drawn to deliver 30 drops per ml, two drops of serum were serially diluted in two-fold steps in precipitation tubes containing two drops of Ca-Mg saline. One drop of complement and one drop of 3 per cent. S.R.C. were then added, and the tubes incubated in a water-bath at 37°C for ½ hr, then placed in the refrigerator at 4°C overnight, and read. Antibody response was measured as the log of the haemolytic titre, obtained by taking the tube numbers as response values (cf. Carlinfanti 1948). The following classes of response were recorded: 5, complete haemolysis; 0, no haemolysis; and the intermediate classes 4, 3, 2, and 1 were graded according to the degree of haemolysis in the tube and the cell deposit at the bottom of the tube. Each response was plotted on a separate card, and the 50 per cent. end-point determined by drawing a regression line through the end-points from the last 5 value to zero. Where, in very low responses, no 5 value was recorded, the regression line was interpolated back to the 50 per cent. end-point. Response values intermediate between whole numbers were taken to the nearest 0.25 of a unit.

Naturally occurring agglutinins to S.R.C. have been found in mice by Lewis and Loomis (1928) and Stern and Davidshon (1954) but no reports of natur-

ally occurring haemolysins have come to our observation. To investigate their possible presence in our mouse stocks, 80 mice were bled prior to injection and their serum tested by the routine method. None were found.

TABLE 1
DIFFERENCES IN RESPONSE DUE TO SEX

Mean Response at	Parents				Offspring			
	♀♀	♂♂	D.F.	<i>t</i>	♀♀	♂♂	D.F.	<i>t</i>
3 days	2.203	1.732	226	1.96*	3.457	0.895	356	12.38***
6 days	8.000	7.255	211	3.49***	8.217	7.296	331	3.44***

* $P < 0.05$.

*** $P < 0.001$.

In order to determine the experimental error of the measurement of haemolytic titre, 57 mice were injected with 0.25 per cent. S.R.C. given in a single dose of 0.5 ml. The mice were bled 6 days later and their sera tested in duplicate for antibody response. The response value in each tube was determined by one operator and recorded by another; a first reading of each response was made prior to the duplicate reading, so that in no case were two readings on any one serum made consecutively. The repeatability was determined by a

TABLE 2
EFFECT OF AGE ON RESPONSE

Response to Age	Parents		Offspring	
	♀♀	♂♂	♀♀	♂♂
Mean age in weeks	25.83	26.34	22.78	19.71
Regression of response on age				
3 days	-0.259 ± 0.023	-0.019 ± 0.064	-0.928 ± 0.24	0.085 ± 0.17
6 days	-0.1667 ± 0.02	-0.034 ± 0.02	-0.485 ± 0.18	0.914 ± 0.33

correlation of the duplicate estimates. This gave a value of $r = 0.99 \pm 0.13$. In view of this high degree of repeatability, single measurements were considered adequate.

In order to choose the best time for injection to bleed the mice, time-response curves, using a selected dose of 0.01 per cent. S.R.C. given in a single intravenous injection, were determined. The time response of 20 animals to this dose is shown in Figure 1. From these data it was decided to bleed at 3 and 6 days.

The frequency distribution of response for 3- and 6-day responses for both sexes is given in Figure 2. The 6-day responses are normally distributed; the distributions of the 3-day responses have an apparent bi-modality owing to the large number of animals which failed to respond.

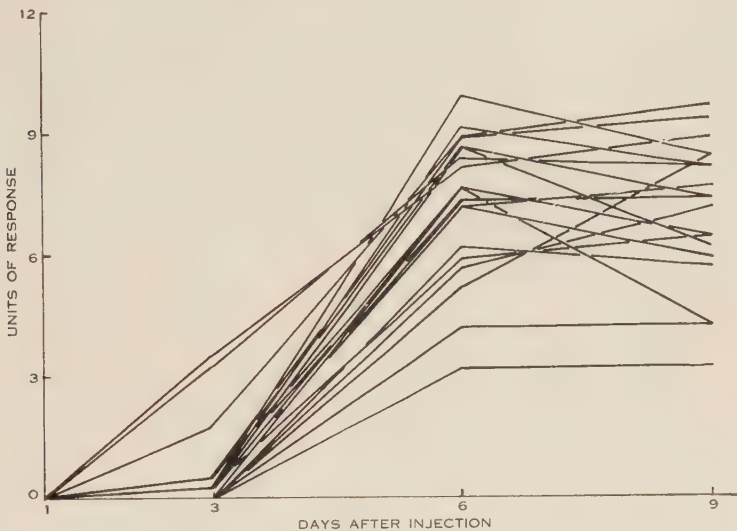


Fig. 1.—Time response curves of 20 mice to a single injection of 0.5 ml of 0.01 per cent. S.R.C.

(c) Results

Before combining all the data it was thought advisable to examine the effect of sex and age on the response of individuals, as the mice used differed considerably in age, and response was measured in both sexes. The difference in response of the two sexes is shown in Table 1 and is marked at both 3 and 6 days. Table 2 shows that the effect of age on response in the parents is marked in females at both 3 and 6 days, the degree of response falling off with increasing age. The effect of age in the males is negligible. In the offspring, which were younger when tested, the effect in females is apparently much stronger, but in males there is an increase in response with increasing age at both 3 and 6 days. It is possible that the ability to respond reaches a peak and falls to an asymptote, the peak being reached much earlier in females. The data examined here are not adequate to decide this point. A similar sex difference of response was found by Gorer and Schutze (1938) to the antigens of *Salmonella typhimurium* and *S. enteritides*.

III. EXPERIMENT 2

(a) Measurement of Heritability

In Table 3 the mean responses of parents and offspring are shown. As would be expected the female offspring which were younger than their female parents when tested have a somewhat higher response at 3 days and a slightly

higher response at 6 days. The males which were also younger than their male parents had a markedly lower response at 3 days and about the same at 6, suggesting that the rather low regression of response on age found at 3 days is more correct than the higher one at 6 days which in any event has a high standard error.

The responses were corrected to constant age and sex and regressions of offspring on mid-parent were calculated using corrected figures. These regressions were 0.2836 ± 0.1475 at 3 days, and 0.1097 ± 0.1165 at 6 days, giving a rather low heritability at both times of sampling. The correlation between the response of a mouse at 3 days and its response at 6 days was found to be $r = 0.565 \pm 0.068$. The heritability of response to S.R.C. is intermediate between that found for response to T.M.V. ($h^2 = 0.87$) and B.P.A. ($h^2 = 0.09$), being nearer that of B.P.A.

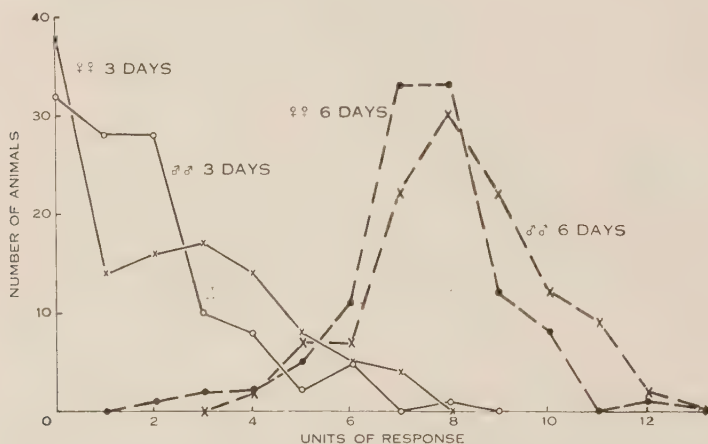


Fig. 2.—Frequency distributions of 3- and 6-day responses of male and female mice to 0.01 per cent. S.R.C. given intravenously.

Besides the inadequacies of measurement discussed in the introduction which, if real, would mask the genetic variation, there are sources of environmental variation affecting measurement which, while not affecting the genetic variation *per se* will increase the non-genetic variation and the error, giving a lower measure of heritability. Of such sources of variation in the present study, those considered of possible importance were measurements made on different days, and the use of S.R.C. of different ages. To determine the significance of the variability introduced by these factors the following factorial experiment was designed. The use of S.R.C. from different sheep was also included although it was not a source of variation in the work just described.

(b) Materials

More anti-serum was required than could be obtained from mice, and for convenience rabbit anti-S.R.C. serum was used. Three rabbits were each given two intravenous injections of 1 c.c. of 1 per cent. S.R.C. spaced by 7 days. They

were bled from the marginal ear vein 7 days after the last injection. After clotting at room temperature for 12 hr the serum was pipetted off, centrifuged, and stored at -20°C .

S.R.C. were obtained from four different sheep. For the purpose of the factorial design of the experiment, the timing of bleeding was so arranged that on two different days, blood of the ages 0, 2, 4, and 6 days would be available from each sheep. Guinea pig serum was used throughout as a source of complement.

TABLE 3
MEAN RESPONSES OF PARENTS AND OFFSPRING

Mean response at	Parent ♀♀	Offspring ♀♀	D.F.	<i>t</i>
3 days	2.203	3.457	286	4.813***
6 days	8.000	8.217	268	0.998
	Parent ♂♂	Offspring ♂♂		
3 days	1.732	0.895	296	4.33***
6 days	7.255	7.296	272	0.163

*** $P < 0.001$.

(c) Methods

The serum from each rabbit was divided into 32 samples and each sample allocated a random number from Fisher and Yates' tables (Fisher and Yates 1953). The samples from each animal were then randomly divided into eight groups of four samples. Four groups of samples were randomly allotted to each of the two different days on which testing was to be made. On each day the four groups of samples were allocated at random to the four different sheep, and the samples in each group were similarly allocated to the four groups of cells of different ages, against which they were to be tested; so that on each of 2 days the serum from three rabbits was tested against red cells of 0, 2, 4, and 6 days of age from four different sheep. The analysis of variance of this factorial experiment is given in Table 4.

(d) Results

As shown in Table 4 the main factors, other than differences between rabbits which introduce significant variation are differences in age of S.R.C., and unspecified differences associated with the day on which a test was made. First order interactions between rabbits and days, and between sheep and S.R.C. of different ages, also introduce a statistically significant variation. The former, while not of any great importance in the present analysis, would have a more marked effect where more rabbits were being compared over a wider range of days. The interaction between sheep and S.R.C. of different ages is shown

in Figure 3. Although response increases with increased age of S.R.C. with a linear trend, the S.R.C. from different sheep show considerable heterogeneity.

The data on which the heritability was estimated were, unfortunately, not suitably recorded to allow a re-estimate of heritability in the light of these findings. It is considered very unlikely, even had this been possible, that the estimate would have reached $h^2 = 0.5$. This is still much lower than that found to T.M.V. and it was decided to examine a multiple antibody system of known constitution in relation to quantitative measurements of response. To this end the following experiment was designed.

IV. EXPERIMENT 3

(a) Materials

Human red cells of groups A and B together with two sources of cells of group AB, referred to as AB_1 and AB_2 , and two sources of anti-A (a_1 , a_2) and anti-B (b_1 , b_2) were kindly supplied by Dr. R. J. Walsh of the Red Cross Blood Transfusion Centre.

TABLE 4

ANALYSIS OF VARIANCE OF FACTORS AFFECTING THE MEASUREMENT OF HAEMOLYTIC TITRE

Source of Variation	D.F.	Mean Square
Rabbits (<i>R</i>)	2	227.14****
Sheep (<i>S</i>)	3	0.42
Ages of S.R.C. (<i>A</i>)	(3)	
Linear	1	8.81**
Quadratic	1	0.40
Cubic	1	0.35
Days (<i>D</i>)	1	1.87**
First order interactions		
<i>R</i> × <i>S</i>	6	0.09
<i>R</i> × <i>A</i>	6	0.05
<i>R</i> × <i>D</i>	2	1.23*
<i>S</i> × <i>A</i>	9	1.37***
<i>S</i> × <i>D</i>	3	0.07
<i>A</i> × <i>D</i>	3	0.14
Error	57	0.26

* $P = 0.05-0.01$.*** $P < 0.001$.** $P = 0.01-0.001$.**** $P < < 0.001$.

(b) Methods

a_1 and b_1 were mixed and are referred to as ab_1 , and similarly a_2 and b_2 were mixed and referred to as ab_2 . ab_1 and ab_2 were then each divided into three volumes; the first was left unabsorbed, the second absorbed with cells of group B, and the third absorbed with cells of group A. Each of these six groups of antiserum was then made up in four strengths, 1 (undiluted), $\frac{1}{2}$, $\frac{1}{4}$,

and $\frac{1}{2}$. Each of these 24 lots of antisera was then tested for its ability to agglutinate both AB₁ and AB₂ cells. The whole process was then repeated making a total of 96 agglutination estimations.

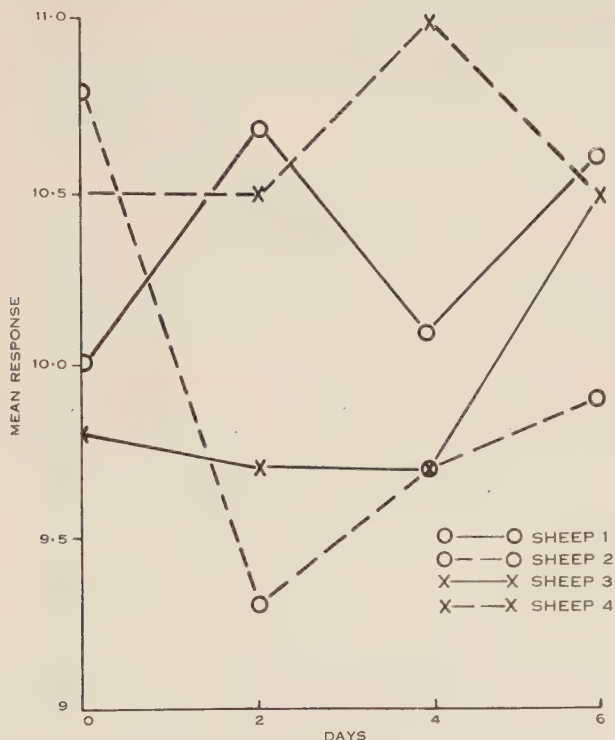


Fig. 3.—First order interaction showing the heterogeneity of mean responses to S.R.C. of different ages from different sheep.

The titre was converted to a log scale by reading tube numbers as response values. Four grades of agglutination were scored, namely 3 complete agglutination, 0 no agglutination, and 2 and 1 intermediate degrees of agglutination. The 50 per cent. end-points were determined by a discriminant analysis (Claringbold, Sobey, and Adams, unpublished data). The analysis of variance, using these end-points, is given in Table 5.

(c) Results

The titres of the ab_1 and ab_2 sera are significantly different, as would be expected, but their analogous behaviour in respect to treatment is borne out by the absence of significant interactions. The titre of a_1 was higher than b_1 and that of a_2 higher than b_2 ; as both differences are of a similar order, the data can be pooled. Hereafter, and in Table 5, the mean titres of b_1 and b_2 , a_1 and a_2 , ab_1 and ab_2 are referred to as b , a , and ab respectively. When ab serum

is absorbed with B cells the *a* antiserum left has a titre which is exactly the same as that of unabsorbed serum. On the other hand, when *ab* serum is absorbed with A cells the remaining *b* antiserum has a titre well below that of whole serum. (Fig. 4 and Table 5.) This result was found at all dilutions and shows that the titre of *a* antiserum has been measured throughout and the presence of *b* has gone undetected.

These data illustrate that with a mixture of two antibodies the titre of the mixture as measured by agglutination is the same as the titre of the component antibody of highest titre.

TABLE 5
ANALYSIS OF VARIANCE
50 per cent. end point titre

Source of Variance	D.F.	Mean Square	<i>F</i>
Biological			
Source of <i>ab</i>	1	1.17	23**
Source of AB	1	0.03	<1
Treatments	(2)		
<i>ab</i> × <i>a</i>	1	0.03	<1
<i>ab</i> + <i>a</i> × <i>b</i>	1	44.56	873***
Interactions as error	7	0.051	
Experimental			
Replication	1	0.08	<1
Dilutions	(3)		
Linear	1	86.02	331***
Quadratic	1	0.00	
Cubic	1	0.51	2.0
First order interactions	19	0.23	<1
Experimental error	61	0.260	

** $P=0.01-0.001$.

*** $P<0.001$.

V. DISCUSSION

The heritability of response to S.R.C. is intermediate between that found for response to T.M.V. and B.P.A., being probably nearer that of B.P.A. (Sang and Sobey 1954). It is difficult to see why response to T.M.V. should be so much more highly heritable than to the other two antigens. The experiments carried out to investigate the extent of environmental variation did not indicate that this variation would affect heritability to a significant extent. It is always possible that the genetic variation of response to T.M.V., which appears to be a single response under the conditions used (Sang and Sobey 1954), is, in fact, greater than the genetic variation of response to B.P.A. and S.R.C. However, an obvious cause of discrepancy is the method of measuring response, which the test carried out using human red cells shows may be quite inadequate, measuring only the concentration of a single antibody in a mixture, the measurement

being always that of the antibody in greatest concentration. It would appear, therefore, that the methods used to measure antibody response are unable to detect either the total response of a system of multiple antibodies, or the variation of any one component antibody present in the system. Where a single antibody response is concerned, as in T.M.V., they would appear to be adequate.

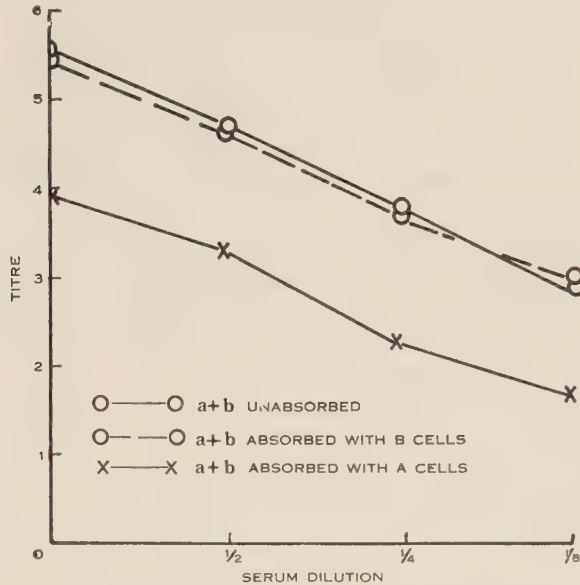


Fig. 4.—Mean titres of absorbed and unabsorbed mixed antisera.

Davidshon and Stern (1954) found differences between inbred lines of mice in their response to S.R.C., but with considerable "within line" variability. This variability would not be expected if the explanation of low heritability of complex antigens put forward here is correct, but since Davidshon and Stern used mice of different age and sex without correction and red cells from different sheep, their results are not comparable with ours. In any event, recent findings by Gruneberg (1954), McLaren and Michie (1954), and Biggers and Claringbold (1954) indicate that the phenotype of inbred lines does not reflect their genetic uniformity.

In conclusion, it is suggested that the present work supports previous contentions that studies concerning the inheritance of antibody response require a detailed knowledge of the complexity of the antigens concerned, and some means of identifying and measuring the different individual antibody responses, or of measuring the total of all the responses present.

VI. ACKNOWLEDGMENT

We are indebted to Dr. P. J. Claringbold for statistical advice.

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